Institut für Veterinärphysiologie
der Vetsuisse-Fakultät Universität Zürich

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Involvement of the histaminergic system in amylin and leptin action – a study with H1rKO mice

Inaugural-Dissertation

zur Erlangung der Doktorwürde der
Vetsuisse-Fakultät Universität Zürich

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Zürich 2011

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Summary

The aim of this study was to investigate the involvement of histamine H1 receptors in the amylin-leptin interaction using H1rKO mice.

Compared with WT controls, 4-5 months old H1rKO mice had a significantly higher body weight, higher amount of fat mass and higher leptin levels; amylin and insulin levels were unchanged. No significant differences were detected in energy expenditure (EE), respiratory quotient or physical activity; however, H1rKO mice showed a tendency toward decreased EE. Food intake (FI) after a 6-hour fast was significantly higher.

H1rKO mice were less responsive to amylin and leptin injections unless very high doses were used. Interestingly, the amylin-induced activation of neurons in the area postrema and the leptin-induced activation of hypothalamic nuclei were not affected, thus the reduced amylin and leptin action in H1rKO mice may not be due to the insensitivity at their primary brain sites.

Finally, WT mice showed reduced FI after the combined amylin-leptin treatment, but H1rKO did not respond.

With this study we showed that the lack of H1 receptors markedly reduced the anorectic effect of amylin and leptin. Since the primary responding brain areas were still responsive, as indicated by cFos and pSTAT3 signaling, the dysfunction of signaling seems to lie in the respective downstream pathways.

Zusammenfassung

Ziel der Arbeit war es, den Einfluss von Histamin H1 Rezeptoren auf die Interaktion von Amylin und Leptin bei H1rKO Mäusen zu untersuchen.

Verglichen mit WT Mäusen zeigten 4-5 Monate alte H1rKO Mäuse ein signifikant erhöhtes Körpergewicht, einen erhöhten Körperfettanteil sowie erhöhte Plasma-Leptinwerte; Amylin und Insulin waren unverändert. Energieverbrauch, respiratorischer Quotient und körperliche Aktivität waren nicht signifikant verändert, trotz eines tendenziell erniedrigten Energieverbrauchs bei H1rKO Mäusen. Weiter zeigten diese Mäuse eine signifikant erhöhte Fasten-induzierte Futteraufnahme.

Amylin und Leptin führten bei H1rKO Mäusen nur in sehr hohen Dosierungen zu einer Verzehrreduktion. Interessanterweise war die Amylin-induzierte Aktivierung von Neuronen in der Area postrema und die Leptin-induzierte Aktivierung gewisser hypothalamischer Kerngebiete nicht vermindert, so dass die reduzierte Verzehrsantwort bei den H1rKO Mäusen nicht auf einer Insensitivität der jeweils primär-rezeptiven Hirnareale zu beruhen scheint. Schliesslich zeigten nur die WT, nicht aber die H1rKO Mäuse eine reduzierte Futteraufnahme nach kombinierter Amylin/Leptin Gabe.

H1 Rezeptoren sind offensichtlich an der Wirkung von Amylin und Leptin beteiligt. Da die entsprechenden primären Hirnareale sensitiv gegenüber Amylin bzw. Leptin bleiben, nehmen wir an, dass eine Signalstörung distal der primären Sensoren vorliegt.

1 Introduction

1.1 Obesity epidemic

Obesity is defined as an excessive amount of body fat in relation to lean mass of sufficient magnitude to produce adverse health consequences. Obesity results from an imbalance between energy intake and energy expenditure. Worldwide there are far more people dying from obesity and its complications, such as metabolic syndrome, insulin resistance and type 2 diabetes, fatty liver disease and cardiovascular disease, than from being underweight or malnourished (Flegal et al., 2007). Currently, over 60% of American adults are overweight (BMI ≥ 25) or obese (BMI ≥ 30) and the obesity prevalence in adults and children keeps on growing dramatically (Zamboni et al., 2005). The World Health Organization illustrated the occurrence of overweight (937 million people) and obesity (396 million people) worldwide in 2005 and estimated that by 2030, the total number of overweight and obese people will reach 1.35 billion and 573 million, respectively (Kelly et al., 2008). In Switzerland, the increase in overweight and obesity between 1992 and 2007 was also enormous; 27'000 cases of type 2 diabetes, 63'000 cases of high blood pressure and 37'000 cases of dyslipidemia could have been avoided if people had not become overweight and obese over those 15 years (Davin et al., 2011).

How to prevent and treat obesity is one of the most discussed topics in our society. Lifestyle change is the least intrusive way to counter obesity and eventually diabetes. However, additional treatments like surgical intervention or pharmacological therapy, e.g. hormonal treatment, are required when the disease worsens and lifestyle changes are no longer sufficient. Currently, surgical intervention is the only proven effective treatment of obesity that results in reduced mortality. Unfortunately, many body weight-lowering drugs have been developed but then again been withdrawn from the market due to unacceptable side effects. Since the central and peripheral mechanisms that play a role in the control of food intake and energy homeostasis are still not fully understood, and since the development of efficient and safe pharmacological treatments of obesity is rather limited, it is crucial to continue to investigate the

physiological processes that control feeding and energy balance.

1.2 Physiological control of food intake

The physiological control of food intake and body weight is maintained by a complex system in which many factors play a role. Energy balance is primarily regulated by neural and hormonal signals that are integrated in the brain (Seeley and Woods, 2003). Of main interest are peripherally-produced hormones, some of which are considered physiological controllers of eating (Fig. 1.1).

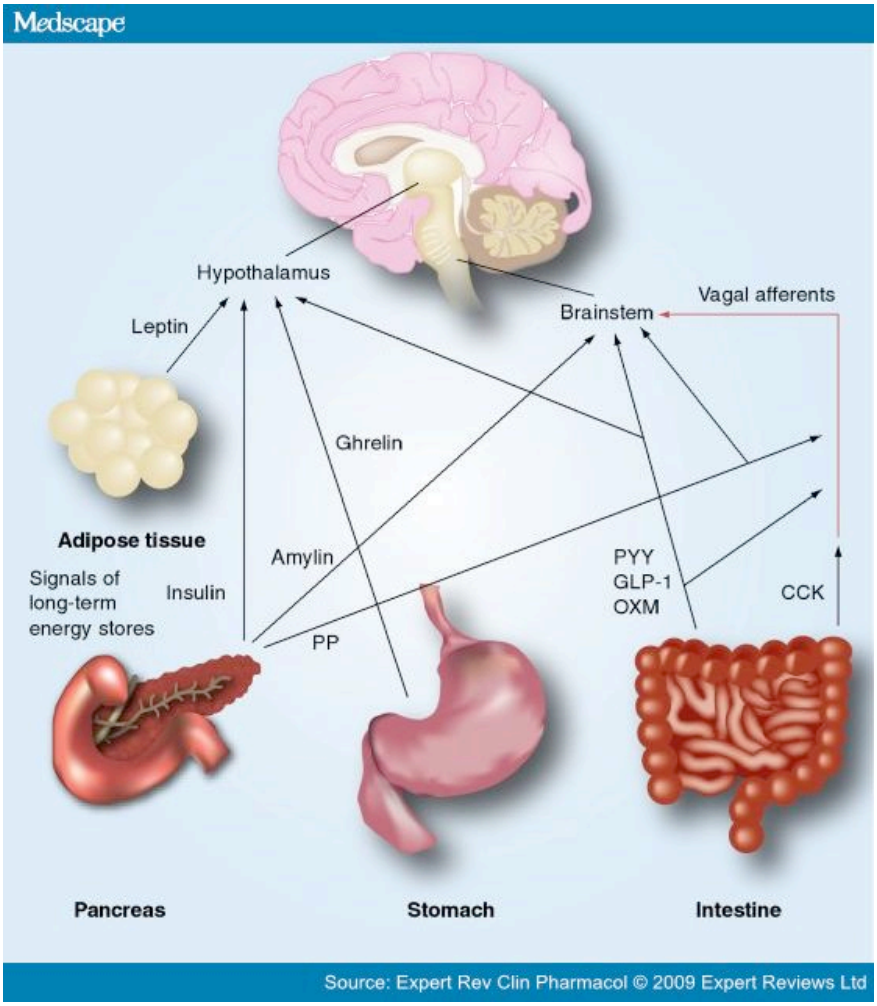

Figure 1.1: A diverse and complex network of circulating hormones and brain centers communicate to control food intake and body weight (Source Medscape: Expert Rev Clin Pharmacol © 2009 Expert Reviews Ltd)

Conceptually, there are two categories of peripheral signals that influence food intake. The first category includes short-term signals generated during meals. Such signals control two meal-related processes, including satiation (the termination of an ongoing meal) and satiety (the interval from the termination of one meal until hunger or the onset of the next meal). By stimulating the hindbrain directly or indirectly via sensory neuronal pathways, satiation signals inhibit food intake. Examples for this first category are cholecystokinin (CCK), amylin, peptide YY (PYY) and glucagon-like peptide-1 (GLP-1) secreted by the intestine and pancreas.

Hormones secreted from the pancreatic islets and the adipose tissues comprise the second category of signals controlling food intake. The circulating levels of these adiposity signals, which include insulin (Lovett and Booth, 1970) and amylin (Morley and Flood, 1991) from the pancreas and leptin (Zhang et al., 1994) from adipocytes, are proportional to the amount of body fat and are thought to have effects on food intake and energy expenditure that control the long-term maintenance of energy homeostasis.

An important anatomical structure for the control of food intake is the hypothalamus - hindbrain axis of the brain. Two hypothalamic regions especially relevant to the control of food intake are the arcuate nucleus of the hypothalamus (ARC) and the ventromedial hypothalamus (VMH). Additionally, regions of the hindbrain, like the area postrema (AP) and the nucleus of the solitary tract (NTS), are also important for the control of energy balance, in particular for meal-associated signals. Afferent fibers of the vagus nerve and visceral nerves via the spinal cord transmit information about the nutrient state to the hindbrain. Additionally, satiation and adiposity hormones circulating in the blood can either act on peripheral nerves (e.g. CCK), cross the blood brain barrier (BBB, e.g. insulin) or bind directly to areas of the brain that lack a complete BBB (e.g. amylin binding to AP).

The ARC contains both insulin and leptin receptors (Plum et al., 2005); their activation influences the expression of various neuropeptides synthesized in the ARC that control feeding behavior. The major output of the ARC is to a pair of neuronal circuits with functionally opposite actions; one providing an anabolic tone and the

other providing a catabolic tone (Seeley and York, 2005). Catabolic neurons in the ARC synthesize pro-opiomelanocortin (POMC), which is processed into α-melanocyte stimulating hormone (α-MSH) and other neuropeptides; α-MSH reduces food intake by binding to melanocortin 3 (MC3R) and melanocortin 4 (MC4R) receptors (Cone, 2005). The anabolic neuronal population in the ARC synthesizes neuropeptide Y (NPY) and agouti-related protein (AgRP) (Elmquist et al., 2005, Morton et al., 2006); they increase food intake by acting on Y receptors and by antagonizing MC3R and MC4R, respectively (Cone, 2005, Stanley et al., 2005). Both populations of ARC neurons project throughout the hypothalamus. Namely, the lateral hypothalamus (LHA) and the paraventricular nucleus (PVN) are richly supplied with NPY/AgRP- and POMC-expressing axons originating from insulin and leptin-sensitive ARC neurons (Elmquist et al., 1998, Elmquist et al., 1999). In fact, the PVN is thought to be the primary site of NPY action (Stanley and Leibowitz, 1984, Billington et al., 1994).

The VMH is another key hypothalamic nucleus controlling energy homeostasis. It has been shown that electrical stimulation of the VMH decreases food intake in food-deprived animals (Anand and Dua, 1955, Oomura et al., 1967), while lesions of the VMH produce obesity and hyperphagia (Hetherington and Ranson, 1983, Bergen et al., 1998). The VMH also contains receptors for several neurotransmitters such as histamine, serotonin, GABA and dopamine, which are important for the control of feeding behavior, energy balance and glucose sensing (King, 2006). Furthermore, the VMH seems to be involved in the metabolic control of eating, e.g. by glucose. The glucostatic theory claims that the VMH is an important locus of glucose receptors that initiate satiety (Mayer, 1955, Marshall and Mayer, 1956). This theory is supported by the fact that a large part of VMH neurons are glucose responsive (Anand et al., 1964, Oomura, 1973, Song et al., 2001, Kang et al., 2004, Levin et al., 2004).

The hindbrain plays an essential role in feeding behavior both by its role as major relay of afferent signals and by its motoneurons. In fact, all signals that control food intake must eventually engage the motor output circuits situated here (Grill, 2006). Regions of the hindbrain also integrate information from circulating hormones and metabolites, and they modulate vagally-mediated information from the gut and

periphery, before relaying this viscerosensory information to the forebrain. The NTS of the dorsal medulla represents the first central terminus for gastrointestinal and gustatory afferent fibers from the periphery (Norgren, 1978). Visceral information is then relayed to regions of the hypothalamus either directly or via the parabrachial nucleus (Herbert et al., 1990). Ascending catecholaminergic (CA) neurons of the NTS, many of which are believed to co-release NPY, target hypothalamic neurons (Sawchenko et al., 1985). The AP lies adjacent to the NTS. It is a circumventricular organ (CVO), and thus lacks a functional BBB (Gross, 1992), located at the floor of the 4th ventricle in the ventral medulla. The AP has reciprocal projections with the NTS and parabrachial nucleus and also receives afferent information from the glossopharyngeal nerve (van der Kooy and Koda, 1983, Shapiro and Miselis, 1985). As the AP receives gut and visceral information through these connections, and is sensitive to circulating signals indicative of the energy status in the internal milieu, it is in a unique position to integrate both circulating and peripheral sensory information, deeming this an important site for the control of autonomic function (Fry et al., 2007).

1.3 Amylin

Amylin is a satiation hormone, which is co-secreted with insulin by pancreatic β-cells in response to the intake of nutrients. During and shortly following food intake, circulating concentrations of both hormones increase significantly. The primary functions of amylin include the inhibition of gastric acid secretion, gastric emptying, pancreatic glucagon secretion, digestive enzyme secretion, and eating. Thus, following food intake, amylin acts to stabilize blood glucose levels and to promote satiation (Young and Denaro, 1998, Reda et al., 2002). Acute peripheral amylin administration reduces food intake dose-dependently, mainly by reducing meal size while having no effect on the following intermeal interval (Lutz et al., 1995).

Amylin's action depends on an activation of the central nervous system namely of the AP, which contains a high density of amylin receptors (Sexton et al., 1994). The role of amylin receptors expressed in other brain sites (Sexton et al., 1994, van Rossum et

al., 1994) is much less clear and their relevance for the control of eating has not been tested.

The amylin receptor is a heterodimer, and all necessary components including the core calcitonin receptor (CT-R) and receptor-activity modifying proteins (RAMPs) 1 or 3 (Christopoulos et al., 1999, Muff et al., 1999) are expressed in the AP. Furthermore it has been shown that AP lesions cause a loss of the anorectic effect of amylin (Lutz et al., 1998) and that local infusion of an amylin antagonist into the AP increases meal size (Mollet et al., 2004). Thus experimental data clearly indicate that the AP is the primary target site for amylin.

Peripheral administration of amylin, at doses that are sufficient to reduce food intake, induces a strong cFos response in the AP and other brain areas. cFos is a nuclear protein used as an indirect marker for neuronal activity (Rowland et al., 1997, Edwards et al., 1998, Rowland and Richmond, 1999, Riediger et al., 2004) and that may be visualized by immunohistochemistry. Hence, by analyzing cFos expression after administration of amylin compared to controls, the relative amylin-induced neuronal activation can be quantified. Amylin induces cFos accumulation not only in the AP, but also in nuclei downstream of the AP, including the NTS, the external lateral parabrachial nucleus (LPBE), the central nucleus of the amygdala (CEA), and the lateral division of the bed nucleus of the stria terminalis (BSTL) (Rowland et al., 1997, Riediger et al., 2004). Importantly, the amylin induced cFos expression in these projection sites is absent in AP lesioned rats. The LPBE, which projects to several hypothalamic nuclei including the ARC and VMH (Li et al., 1994), is also required for full expression of amylin-induced anorexia (Becskei et al., 2007).

1.4 Leptin

Leptin is the protein product of the obese gene (ob) and is primarily secreted by adipocytes of the white adipose tissue. Serum leptin concentrations are therefore directly proportional to the amount of body adipose tissue (Considine et al., 1996) at least under relatively weight stable condition (Gloy et al., 2010). Numerous studies indicate that the ARC is the primary site of leptin action (Baskin et al., 1999, Morton

et al., 2003). It has been shown using in situ hybridization that leptin receptor mRNA is highly expressed in the ARC, with lower levels also detectable in the VMH and the dorsomedial hypothalamus (DMH; Schwartz et al., 1996).

Leptin is structurally related to cytokines (Zhang et al., 1997), and its receptor is member of the cytokine receptor superfamily (Tartaglia et al., 1995). Leptin binds to the long form of the leptin receptor (ObRb) and activates cytokine-like signal transduction cascades by stimulating the receptor-associated janus tyrosine kinase 2 of the JAK-STAT (signal transducer and activator of transcription) pathway; this ultimately results in the phosphorylation of STAT3 (pSTAT3; Zhong et al., 1994, Ihle, 1995, Baumann et al., 1996, Ghilardi et al., 1996, Vaisse et al., 1996, Bjorbaek et al., 1997, White et al., 1997). The activated pSTAT3 is an indirect marker for functional leptin receptor activity. Following leptin administration, pSTAT3 signaling occurs in hypothalamic nuclei including the ARC and the VMH, but also in regions involved in reward mechanisms, including the ventral tegmental area (Fulton et al., 2006, Hommel et al., 2006). Activation of STAT3 by leptin may be involved in changes in gene expression in leptin-responsive nuclei. For instance, stimulation of POMC gene expression in hypothalamic neurons seems to require the leptin-induced phosphorylation of STAT3 (Munzberg et al., 2003).

Both centrally and peripherally administered leptin reduce food intake and increase energy expenditure, and consequently lower body weight when administered chronically (Halaas and Friedman, 1997, Hwa et al., 1997, Widdowson et al., 1997). This effect can be seen best in obese leptin-deficient mice (ob/ob) when leptin is administered because these mice are fully responsive to leptin. This results in reduced hyperphagia and obesity. In contrary, obese mice that are deficient in the leptin receptor (db/db) do not respond to leptin treatment (Halaas et al., 1995, Chen et al., 1996).

Interestingly, obesity in both humans and rodents, with the exception of ob/ob mice, results in high levels of circulating leptin, known as hyperleptinemia, but these elevated levels seem to be unable to reduce appetite or to increase energy expenditure. In other words, common obesity seems to be associated with a state of leptin

resistance, which is in principle comparable to hyperinsulinemia and insulin resistance occurring in many type 2 diabetes patients. The exact cause of the development of leptin resistance is not known, but multiple factors may contribute to the problem. One hypothesis suggests that elevated blood leptin levels may overstimulate leptin receptors. Overstimulation may eventually lead to an upregulation of negative feedback pathways which block further leptin signaling (Knight et al., 2010). This theory is supported by the fact that leptin also stimulates the expression of suppressor of cytokine signaling 3 (SOCS-3), a protein that directly inhibits leptin signaling (Enriori et al., 2007). Moreover, SOCS-3 is a negative regulator of pSTAT3 signaling. Leptin resistance develops most strongly in the ARC and is therefore associated with elevated SOCS-3 levels in the ARC (Munzberg et al., 2004). Heterozygous SOCS-3 deficient mice are more sensitive to the weight-reducing effects of leptin and are resistant to the development of obesity (Howard et al., 2004). Together, these data indicate that excessive activity of SOCS-3 may be a potential mechanism for leptin resistance, and that the inhibition of SOCS-3 expression or function might therefore be a potential target for the development of drugs aimed at improving leptin sensitivity (Bjorbaek et al., 1998).

Two additional theories purport to explain how rodents become leptin resistant. The first suggests that peripheral leptin becomes unable to reach its target in the brain due to saturation of the transporting system across the blood brain barrier. This may result in a reduced ratio of peripheral leptin to leptin levels in the cerebrospinal fluid (Caro et al., 1996, Banks and Farrell, 2003). The second theory states that leptin resistance is due to defects in CNS pathways that facilitate leptin's effect. For instance, diet-induced obesity (DIO) in rodents, which results in decreased leptin sensitivity, impairs the ability of leptin to activate hypothalamic signaling, such as the phosphorylation of STAT3 (El-Haschimi et al., 2000, Levin and Dunn-Meynell, 2002).

To investigate leptin resistance, DIO rodents are excellent models to clarify how leptin signaling becomes compromised when leptin resistance develops. C57BL/6J mice fed a high-fat diet (HFD) exhibit increased body adiposity along with other characteristics of human obesity, such as diabetes mellitus (>70% of mice on HFD)

(Burcelin et al., 2002). The development of obesity and leptin resistance in C57BL/6J mice kept on a HFD can be subdivided into two different stages of leptin resistance. In the early stage of obesity, mice are resistant to peripherally administered leptin. After long-term exposure to HFD for approximately 20 weeks, mice also become resistant to centrally applied leptin (El-Haschimi et al., 2000, Knight et al., 2010). In respect to the potential causes of leptin resistance, this time course suggests that a defect in BBB transport of leptin may have occurred earlier than defects in post-receptor signaling.

The occurrence of leptin resistance in both obese rodents and humans clearly demonstrates that leptin resistance is the main hurdle of leptin-based therapy. Investigations into the reversal of leptin resistance present some promising new avenues for obesity treatment. For instance, combined hormone therapies, such as amylin and leptin co-treatment, appear to enhance leptin function even in obese rats and humans.

1.5 Amylin-leptin synergism

Growing evidence demonstrates that amylin and leptin work synergistically, and not just additively (Trevaskis et al., 2008), to promote a greater decrease in body weight and food intake than either hormone alone (Roth et al., 2010). This effect has been observed in both lean and obese rats, as well as in clinical trials in humans.

In lean rats, acute administration of central leptin increases the acute anorectic effect of peripheral amylin (Osto et al., 2007), while chronic peripheral administration of both hormones leads to a synergistic reduction in body weight and epididymal fat mass (Turek et al., 2010). Additionally, 14-day co-treatment with amylin and leptin actually appears to restore leptin sensitivity in leptin-resistant DIO rats (Roth et al., 2008). When co-treatment is extended to 28 days, DIO-prone rats show a marked decrease in food intake, which results in loss of body weight and a reduction of fat mass; this is significantly greater than in pair-fed control rats (Trevaskis et al., 2008). Therefore, these results suggest that co-administration of amylin and leptin not only

decreases food intake, but also increases energy expenditure with a shift toward fat utilization rather than carbohydrate (Trevaskis et al., 2008).

Similar effects have also been observed in humans. The amylin analogue pramlintide produce significant reductions in body weight and food intake when administered alone in obese humans (Hollander et al., 2004, Smith et al., 2007), but is even more effective when combined with the leptin analogue metreleptin (Roth et al., 2008).
The mechanism underlying the interaction of these two hormones is not fully understood. However, it has been suggested that amylin and leptin act via common neuronal pathways (Turek et al., 2010). While it cannot be ruled out at this time, the AP does not appear to be the primary site of this interaction, because single injections of leptin do not enhance the cFos expression induced by amylin in the AP (Turek et al., 2010). Instead, evidence points to the VMH as a potential site of interaction for amylin and leptin. Amylin treatment restored leptin-sensitivity in DIO rats, as substantiated by an increase in leptin-induced pSTAT3 within the VMH (Roth et al., 2008, Turek et al., 2010). Amylin administration over 7 days also enhanced the pSTAT3 response to leptin in the VMH of lean rats (Cole et al., 2007, Trevaskis et al., 2008). Leptin binding, as determined by receptor autoradiography in the rat brain, was increased in the ARC and VMH by combination treatment with amylin and leptin, and it was also increased by amylin alone in the VMH and the DMH (Turek et al., 2010). Furthermore, amylin knockout mice exhibit reduced leptin receptor expression and attenuated leptin-induced pSTAT3 activity in the hypothalamus, suggesting an important role of amylin for normal leptin signaling, and potentially for the proper development of the leptin system (Roth et al., 2010, Trevaskis et al., 2010b, Turek et al., 2010).

1.6 Histamine and hypothalamus

Another network thought to be involved in the control of food intake and body weight is the histaminergic system. Histamine is produced both in the periphery and in the brain, but only histamine produced centrally can act in the brain because circulating histamine is not able to penetrate the BBB.

Central histamine is produced exclusively by neurons in the tuberomamillary nucleus of the hypothalamus (TM) and surrounding areas (Wada et al., 1991). Histaminergic neurons arising from the TM receive inputs mainly from the limbic system, and project widely throughout the brain (Panula et al., 1984, Watanabe et al., 1984, Airaksinen and Panula, 1988, Inagaki et al., 1988, Ericson et al., 1991). Studies in rats have shown that physiological central histamine release depends on a circadian rhythm and gradually increases during the second half of the light period (Mochizuki et al., 1992), reaching its maximum during the active (dark) phase, i.e. when animals are eating (Morimoto et al., 2001). Hypothalamic histamine is also released in response to feeding (Valdes et al., 2005). This effect is enhanced after a 24-hour fast in rats allowed to eat for 15 minutes (Itoh et al., 1991). Interestingly, histamine release increased not only during actual eating but also during food presentation, i.e. when food was covered and placed in the cage for 15 minutes (Itoh et al., 1991). Furthermore, taste information activates hypothalamic histamine release via the chorda tympani. For example, oral administration of sweet solutions such as sucrose (caloric) or saccharin (non-caloric) produced a decrease in histamine release, whereas oral administration of NaCl and HCl induced a significant increase in histamine release in freely-moving (Treesukosol et al., 2005) but also in anesthetized rats (Treesukosol et al., 2003).

In addition to its effects on histamine release, feeding has also been found to modulate the level of the histamine H1 receptor expression; whole-brain H1 receptor concentration is inversely correlated with voluntary food intake in rats (Haq et al., 1996). Three different types of histamine receptors are expressed in the brain (Deng et al., 2010). The H1 and H3 histamine receptors are thought to be involved in the control of food intake, while H2 receptors mediate other histamine function (Brown et al., 2001). Food intake is suppressed by either activation of the histamine H1 receptor or by inhibition of the histamine H3 autoreceptor (Sakata et al., 1997, Morimoto et al., 1999). H3 autoreceptor activation reduces histamine release from its nerve terminals, and thioperamide, which blocks the H3 receptor, increases histamine release and reduces food intake when infused into the third cerebroventricle (Ookuma et al., 1993).

This research project focused on the central histamine H1 receptor, which seems to play an important role in feeding control (Sakata et al., 2003). The relevance of the H1 receptor was first discovered after observations that some antidepressants and antipsychotic drugs produced side effects like appetite stimulation and weight gain in humans (Kalucy, 1980, Russ and Ackerman, 1988). It was then discovered that these drugs have H1 receptor blocking activity (Hill and Young, 1978, Taylor and Richelson, 1980). Histamine H1 receptors are expressed widely throughout the brain, but especially within the hypothalamus, with a very high density in the VMH (see Fig. 1.2), and more moderate distribution in the DMH and PVN (Palacios et al., 1981).

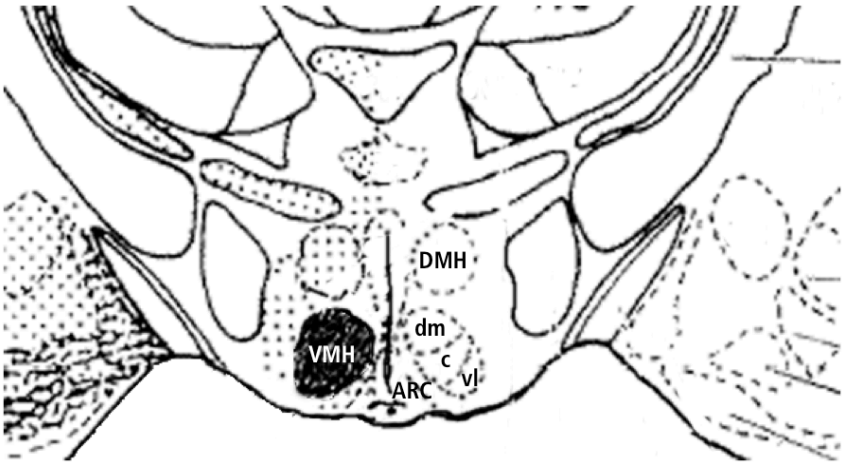

Figure 1.2: Histamine H1 receptors density in the hypothalamus (Palacios et al., 1981). The darker the shading indicates the higher the H1 receptor density in the individual areas; ARC: arcuate nucleus; DMH: dorsomedial hypothalamus; VMH: ventromedial hypothalamus; dm: dorsomedial, c: central, vl: ventrolateral part of the VMH

Activation of central H1 receptors by histamine or H1-agonist administration leads to a suppression of food intake, whereas blockade of the H1 receptors results in hyperphagia and ultimately in obesity (Fujise et al., 1993, Sakata et al., 2003), (Itowi et al., 1988, Lecklin et al., 1998). Similarly, when the histamine concentration is elevated by peripherally injecting L-histidine, a precursor of histamine which is able to penetrate the blood brain barrier, food intake is decreased as well (Morimoto et al., 2001).

There are two established methods that reliably abolish the effect of H1 receptors: administration of H1 receptor antagonists (Mercer et al., 1994) and use of the H1 receptor knock out (H1rKO) mice. H1rKO mice act as a good research model to investigate the functional role of H1r deficiency. Another, though rather unspecific, way to inhibit H1 receptor function, is by using α-fluoromethylhistidine (FMH), which blocks histamine synthesis by irreversibly inhibiting histidine decarboxylase, the enzyme that converts histidine to histamine. As a consequence, H1 receptors are not activated. While all models have been commonly used to investigate the role of the histaminergic system in the control of eating, FMH is currently not available on the market.

1.7 H1rKO mouse model

Treatment with histamine H1 receptor agonists might be an effective and novel approach of therapy against obesity and related metabolic disorders (Masaki et al., 2004). For that reason, a better understanding of H1 receptor function is necessary. Therefore we used H1rKO mice to investigate the role of the histamine H1 receptors in the control of eating in the current study. The H1rKO mouse is an excellent model to investigate the specific function of the H1 receptor and its interaction with hormones that control energy homeostasis and feeding behavior.

H1rKO mice show a significantly higher growth rate and body weight compared to WT mice from about 6 months of age, and they exhibit higher plasma leptin and insulin levels when tested at 48 weeks of age (Masaki et al., 2001b, Masaki et al., 2004). Another study in our laboratory showed that H1rKO mice demonstrate significantly higher body weight already by the age of 12 weeks (Mollet et al., 2001). This indicates that histamine may be involved in aging-related obesity (Tsuda et al., 2002, Masaki et al., 2004). It has also been shown that prior to the onset of obesity, H1rKO mice exhibit an altered circadian rhythm, which leads to abnormal feeding behavior relative to WT controls (Masaki et al., 2004). Moreover, H1rKO mice show increased locomotor activity especially during the early portion of the light phase, but decreased exploratory behavior and more frequent defecation and urination in a new environment (Inoue et al., 1996, Masaki et al., 2004). Because it is known that

disturbances in the circadian feeding rhythm are a risk factor for the development of obesity, such abnormalities in the H1rKO mice may perhaps contribute to their obese phenotype (Beck et al., 1990, Fukagawa et al., 1992, Murakami et al., 1995).

An interesting feature of the H1rKO mice model is the decreased responsiveness to certain hormones that control food intake. Acute amylin (5 and 20μg/kg) and leptin (1.3mg/kg) administration in H1rKO mice results in a markedly attenuated anorectic effect compared to WT controls (Morimoto et al., 1999, Morimoto et al., 2000, Mollet et al., 2001), but this is not observed following CCK (20μg/kg) administration (Morimoto et al., 1999, Morimoto et al., 2000, Mollet et al., 2001); hence, the effect seems specific to only certain hormones involved in feeding control. Further, H1rKO mice treated continuously with leptin over 7 days, either centrally or peripherally, also demonstrate decreased leptin sensitivity, in that they exhibit blunted body weight loss and less food intake reduction compared to wild type mice (Masaki et al., 2004). H1rKO mice also show a decreased cFos response in the hypothalamus following leptin injection (Masaki et al., 2004); these results do not, however, indicate if this leptin resistance in the H1rKO mice is a result of a defective leptin receptor function or of a disruption in the networks downstream of the leptin receptor.

1.8 Interaction between amylin, leptin and histamine

Based on these findings, H1 receptors and the histaminergic system appear to play an important role for the control of feeding, and they seem to be specifically involved in the mediation of the individual effects of leptin and amylin. Here, we hypothesized that the synergistic interaction that exists between amylin and leptin (Osto et al., 2007, Roth et al., 2008, Roth et al., 2010, Trevaskis et al., 2010a, Turek et al., 2010) may also depend on the histaminergic system, and specifically on H1-receptor-mediated effects.

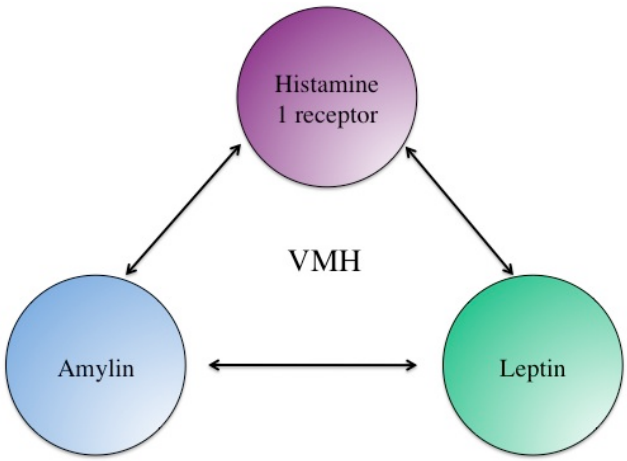

Figure 1.3: This dissertation begins to explore the possible link between the central histaminergic system and amylin and leptin synergy. Experimental evidence points to the VMH as a potential key locus for both histamine action and the interaction between amylin and leptin

The evidence outlined above supports the idea that the histaminergic system may be an important player in the synergistic effects of amylin and leptin on body weight and food intake. As mentioned, H1rKO mice exhibit an attenuated anorectic response to amylin (Mollet et al., 2001). Further studies have shown that amylin's inhibitory effect on food intake is markedly reduced when the specific H1 receptor antagonists chlorpheniramine (CPA) or pyrilamine (PYR) are administered directly into the VMH in rats (Mollet et al., 2003). Additional findings that support a link between amylin and histamine demonstrate that blockade of histamine release by H3 agonists reduces amylin's anorectic effect (Lutz et al., 1996) and that amylin-expressing neuronal fibers colocalize with histamine-producing neurons within the TM, suggesting that amylin function may perhaps involve an interaction with the histamine system (D'Este et al., 2001).

In regards to the link between leptin and histamine function, we already mentioned that mice deficient in H1 receptors show decreased responsiveness to both acute and chronic leptin treatment (Morimoto et al., 2000, Mollet et al., 2001, Morimoto et al., 2001, Masaki et al., 2004). Moreover, Morimoto and colleagues showed that the leptin-induced suppression of food intake is abolished when mice or rats were injected with FMH to block histamine production (Morimoto et al., 1999, Toftegaard et al., 2003). Further, leptin may facilitate hypothalamic histamine release

(Yoshimatsu et al., 1999, Morimoto et al., 2000) because hypothalamic histamine levels increase immediately after leptin (1.3mg/kg i.p.) administration and remain elevated for up to 4 hours (Morimoto et al., 2000), hence at a time when leptin typically exerts its anorectic action.

Finally, as summarized above, the synergistic relationship between amylin and leptin treatment is potentially a very powerful therapeutic tool to treat obesity. Amylin enhances or restores the efficacy of leptin treatment in lean and obese rodents, as well as in humans. Using pSTAT3 as a marker for leptin receptor activation, it appears that the VMH may be a critical site where amylin treatment enhances leptin signaling (Roth et al., 2008). Given the high density of H1 receptors in the VMH (Palacios et al., 1981), and the known effects of histamine in the VMH on food intake (Sakata et al., 1988, Ookuma et al., 1993, Mollet et al., 2003, Magrani et al., 2004), it seems plausible to propose that the histaminergic network acting in the VMH may contribute to the interaction observed between amylin and leptin.

Based on these data, we suggest that these three components, amylin, leptin, and histamine, interact at the level of the VMH to promote a decrease in food intake and body weight. We therefore hypothesized that the histaminergic system, and specifically H1 receptors, may be a critical component contributing to the synergistic relationship between amylin and leptin.

1.9 Approach and hypothesis

The main goal of this dissertation was to determine the role of the H1 receptor in mediating the effects of acute amylin and leptin administration, both independently and in combination, on food intake. Additionally, we sought to more thoroughly describe the metabolic phenotype of H1rKO mice, which was the animal model used for all studies described herein.

1.9.1 Experiment 1: Metabolic phenotyping of H1rKO mice

The first aim was to better characterize the metabolic phenotype of the H1rKO mice.

While some general phenotyping had been performed in these mice, some parameters, such as body composition and energy expenditure (EE) have not been assessed. We therefore measured body composition (i.e. fat mass distribution) via computer tomography, energy expenditure (EE) and respiratory quotient (RQ) via indirect calorimetry, assessed physical activity (PA), and measured daily and fasting-induced food intake (FI) as well as plasma levels of amylin, leptin and insulin, in H1rKO compared to WT mice.

1.9.2 Experiment 2: Do mice lacking H1 receptors respond to higher doses of amylin?

It is known that H1rKO mice are less responsive to acute amylin (5µg/kg or 20µg/kg) treatment (Mollet et al., 2001). Since it was not clear if this also holds true for higher doses, our intention was to assess the action of amylin on food intake in a dose-dependent manner and over a wide dose range in H1rKO compared to WT mice.

1.9.2.1 Does amylin induce cFos activation in the AP of H1rKO mice?

We further wanted to test whether the reduced responsiveness of H1rKO mice to single amylin administration is related to a disruption of amylin signaling in the AP or in downstream projection sites. To test this, amylin at a dose of 50µg/kg was injected intraperitoneally and an immunhistochemical study was conducted to quantify the activation of neurons in the AP by visualizing cFos expression.

1.9.3 Experiment 3: Do mice lacking H1 receptors respond to higher doses of leptin?

H1rKO mice are also less responsive to leptin at a dose of 1.3mg/kg (Morimoto et al., 1999, Mollet et al., 2001). Since it is not clear if this is also true for higher doses, the intention was to assess the individual action of leptin on food intake in a dose-dependent manner over a wide dose range in H1rKO compared to WT mice.

1.9.3.1 Does leptin induce pSTAT3 activation in the VMH and ARC of H1rKO mice?

We further wanted to test whether the reduced responsiveness of H1rKO mice to single leptin administration is related to a disruption of leptin signaling within the leptin-sensitive brain sites, e.g. the VMH and ARC, or in projection sites. To test this, leptin at a dose of 1.3mg/kg was injected intraperitoneally and an immunhistochemical study was conducted to assess the function of leptin receptors by visualizing pSTAT3 signaling in the VMH and ARC.

1.9.4 Experiment 4: Do mice lacking H1 receptors respond to combined amylin – leptin administration?

Because amylin is able to restore leptin's eating inhibitory effect in leptin-resistant rats and humans, and because amylin is able to reinstate leptin-induced pSTAT3 activity in the VMH of DIO rats (Roth et al., 2008), we wanted to investigate whether co-treatment with amylin and leptin is as effective at reducing food intake in H1rKO mice as in WT mice. This experiment addressed the potential role of the H1 receptor in the mediation of the amylin—leptin interaction.

2 Material and methods

2.1 Animals and housing conditions

For all experiments, male C57BL/6J wild type (WT) mice and male histamine H1 receptor knock out (H1rKO) mice were used. The H1rKO mice were generated by homologous recombination (H1rKO; background strain C57BL6) (Inoue et al., 1996). The C57BL/6J mice were either taken from our in-house breeding colony or ordered from Charles River Laboratories (Sulzfeld, Germany) when additional mice were needed; our in-house colony was originally derived from breading pairs supplied by Charles River.

Mice were maintained in a temperature-controlled environment (21±2°C), on a 12h/12h light-dark cycle (lights on at 0100h and off at 1300h). Water and food (see section 2.2 for details) were accessible *ad libitum*, unless otherwise described. Animals were habituated to the housing conditions for at least one week prior to the start of an experiment. Mice were handled daily for the first 2-4 weeks after their arrival with a special desensitization technique (see supplemental section 2.13). Body weight was measured every 1-3 days during the entire experimental time period, and baseline food intake was measured weekly or, when the mice were in experiments, more frequently at specific time points.

All experiments were approved by the Veterinary Office of the Canton of Zurich, Switzerland.

2.1.1 Housing conditions in Makrolon cages

For most experiments, animals were group housed in Makrolon cages (2-5 mice/cage). Occasionally, some mice were single housed due to aggressive behavior.

2.1.2 Housing conditions in hanging wire-mesh cages

For some experiments in which individual food intake was measured, mice were individually housed in hanging, stainless steel wire-mesh cages (24 × 25 × 18cm).

2.1.3 Housing conditions in the metabolic cages

For some feeding trials and for all experiments measuring energy expenditure, mice were individually housed in transparent plastic metabolic cages (24.2 × 16 × 18.4cm) containing wood shavings, paper for nesting and a small carton house. The cages allow the measurement of several metabolic parameters, including oxygen consumption, carbon dioxide production, food and water intake (DietMax, AccuScan Instruments, Inc. Columbus, Ohio 43228, USA). The animals received the standard chow food (see section 2.2) of a comparable composition in powder form as they had received the weeks before in the hanging wire mesh cages. In order to accurately measure physical activity, houses and nesting material were removed during such testing periods.

The water bottles and the food cups were placed on scales for continuous monitoring of drinking and eating. Water and food intake were measured every five minutes using Integra ME Version 2.21 (AccuScan Instruments), as described in detail in section 2.5.

2.2 Experimental diets

The Standard pelleted chow (D3436, Extrudate) and the Powered chow diet (D3433, GLP) had the same composition and were produced by Provimi Kliba AG, Kaiseraugst, CH.

Major Nutrients:

Dry matter	88.0%
Crude protein	18.5%
Crude fat	4.5%
Crude fiber	4.5%
Crude ash	6.3%
NFE	54.2%
Starch	35.0%
Metabolizable energy	13.2MJ/kg

2.3 Amylin and leptin

Amylin (Bachem AG, Bubendorf, CH) was diluted in 0.9% NaCl (Fresenius Kabi AG, Stans, CH) in various concentrations. Recombinant murine leptin (PeproTech EC Ltd, London, UK) was diluted in *aqua ad injectabila* (Ampuwa, Fresenius Kabi AG) in various concentrations. All peptides were freshly dissolved immediately before the start of an experiment and then kept chilled on ice until injection.

2.4 Feeding test procedure

Prior to experiments in which food intake was measured, mice were fed either *ad libitum* (Experiment 2a) or fasted for 6 hours prior to injection at dark onset (Experiment 2b, 3 and 4). For each feeding trial, WT and H1rKO mice were randomly divided into four (Experiment 2a, 3 and 4) or five (Experiment 2b) treatment groups, respectively (see section 2.11). A crossover design was used with randomized order of treatment so that each animal received all treatments and acted as its own control. There was at least one day between two trials to allow for animal recovery and clearance of exogenously administered substances. Food was presented just after the injections at dark onset (1300h). Food intake was typically measured one, two and four hours after injection, as described below.

2.5 AccuScan system in the metabolic cages

The measurement of the gas exchange was achieved by using an Open Circuit Calorimetry system (AccuScan Instruments). The plastic cages were equipped with tight-fitting covers and an inserted pump and gas analyzers, which enabled the measurement of the oxygen (O_2) and carbon dioxide (CO_2) content of air flowing in and out of the cage. Changes of the gas composition of the in-flowing and out-flowing air were indicative of oxygen consumption and carbon dioxide production, respectively. The oxygen analyzer utilized a Zirconia sensor, the carbon dioxide analyzer used a dual beam sensor. Both analyzers were connected to the individual cages and transmitted the measured data to the metabolic integrating system, which also received the data from the food and water scales. Oxygen and carbon dioxide concentrations of each cage were measured for 30 seconds every five minutes. Before the start of an experiment, the analyzers were calibrated; the percentage of oxygen in the room air was set to 20.94%, and by using a 12-hour-fasted WT mouse, the respiratory quotient was adjusted to approximately 0.7. From the measured data, several physiological parameter were calculated by the fusion software (Integra ME Version 2.21, AccuScan Instruments) including O_2 consumption (VO_2), carbon dioxide production (VCO_2), energy expenditure (EE), and respiratory quotient (RQ):

Oxygen consumption:

$$VO_2\,[\text{ml/kg/min}] = \frac{\text{Flow [ml/min]} \times (V_1 + V_2)}{100 \times \%N_2\,\text{Ref} \times \text{Weight [kg]}}$$

$$\%N_2\,\text{Ref} = 100 - (\%O_2\,\text{Ref} + \%CO_2\,\text{Ref})$$
$$V_1 = \%N_2\,\text{Ref} \times \%O_2\,\text{Change}$$
$$V_2 = \%N_2\,\text{Ref} \times (\%O_2\,\text{Change} - \%CO_2\,\text{Change})$$

Carbon dioxide production:

$$VCO_2\,[\text{ml/kg/min}] = \frac{\text{Flow [ml/min]} \times \%CO_2\,\text{Change}}{\text{Weight [kg]} \times 100}$$

The VCO_2 and VO_2 values were used to calculate energy expenditure according to Weir (Weir, 1990):

$$EE\ [kcal/kg/min] = \frac{3.9 \times VO_2 + 1.1 \times VCO_2}{1000}$$

The respiratory quotient was used to identify the preferred source of oxidation; RQ equals 1.0 if carbohydrates are used as energy source; RQ equaling 0.7 indicates if fat is used as the sole energy source:

$$RQ = \frac{VCO_2}{VO_2}$$

2.6 Activity measurement

The physical activity of the mice was measured using a frame of horizontal infrared beams. The number of beam breaks was quantified as a direct measure of activity.

2.7 Blood sampling

Prior to blood sampling, mice were food-deprived for 4 hours. Blood sampling was started at dark onset (1300h), and was conducted as a terminal experiment. Mice were deeply anesthetized with pentobarbital (pentobarbital sodium, 80mg/kg i.p., Kantonsapotheke Zurich, CH), immediately following which the jugular vein was severed with a sharp scalpel, blood was collected in a 500µl EDTA-coated tube (Microvette 500K3E, Sarstedt AG & Co., Nümbrecht, Germany) and mixed with 5µl of a protease inhibitor cocktail (Aprotinin, P2714 Protease Inhibitor Cocktail, Sigma-Aldrich, Buchs SG, CH) to stop the enzymatic degradation of peptide hormones. The blood remained cooled on ice until it was centrifuged for 10 minutes at 1000g. Plasma was transferred to clean Eppendorf tubes and stored at - 20°C until use.

2.8 CT scan for body composition analysis

Body adiposity was determined in sacrificed animals by computerized tomography

(CT) using the La Theta LCT-100 (Aloka CT, Tokio, JP; for validation details, please see (Hillebrand et al., 2010)). The X-ray source tube voltage was set at 50kV with a constant 1mA current. The ice-cooled carcasses were placed supine in Plexiglas holders with an inner diameter of 48mm, resulting in a pixel resolution of 100µm. First, a sagittal image of the entire animal was made to ensure proper placement in the holder and to set the scan area, which was defined from diaphragm to hip joints. La Theta 2.10-Aloka software was used to calculate the volumes of adipose tissue, bone, air, and remaining tissue, using differences in X-ray density. Intraabdominal and subcutaneous adipose tissues were distinguished based on the detection of the abdominal muscle layers (see Figure 3.1; Kobayashi et al., 2002). This automated classification often required manual image-by-image correction. The amount of adipose tissue located between the diaphragm and the hip joints was measured to compare subcutaneous and intraabdominal body fat content in both genotypes. Adipose tissue weights were then computed using the commonly used density factor of 0.92g/cm^3 (Hill et al., 2007) to determine the absolute values in gram.

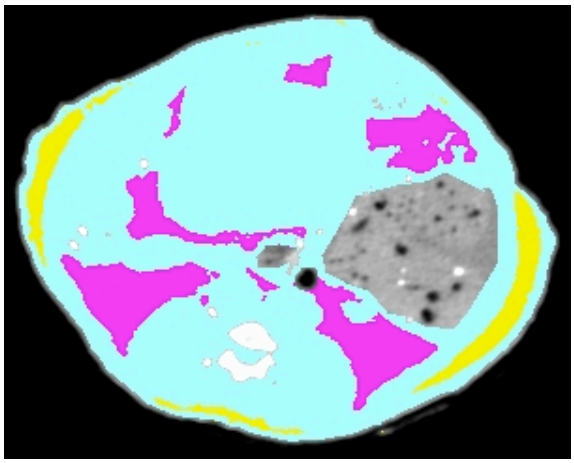

Figure 2.1: Abdominal cross section of a H1rKO mouse; subcutaneous fat (yellow), intraabdominal fat (purple), lean tissue (turquoise)

2.9 Hormone analysis

Amylin, leptin and insulin were measured using plasma samples and the hormone kit Milliplex Mouse Endocrine Immunoassay MENDO-75K-03 (Millipore Corporation,

Billerica, MA, USA). The frozen plasma samples were thawed, mixed and centrifuged at 3000g for 5 minutes. The assay was based on conventional sandwich assay technology and permitted simultaneous detection of multiple hormones from a single sample. Briefly, according to the manufacturer's protocol, the microspheres were incubated with standards, controls and the plasma samples (10µl) in a 96-well microtiter filter plate on a plate shaker overnight (16-18 hours) at 4°C. After incubation, the plate was washed three times with assay buffer (200µl/well) to remove excess reagents (by vacuum between each rinse), followed by the addition of the detection antibody (50µl/well). Then the plate was placed again on the plate shaker for 60 minutes at room temperature, after which 50µl streptavidin-phycoerythrin was added to each well containing 50µl of detection antibody cocktail and incubated for an additional 30 minutes. After a final washing step (3 rounds of washing and fluid removal by vacuum filtration), the beads were re-suspended in buffer (100µl of sheath fluid) and the plate was analyzed using the Bio-Plex analyzer powered by xMAP Luminex technology (Bio-Rad Laboratories Inc., Reinach BL, CH) to determine the concentration of amylin, leptin, and insulin. Data were analyzed by Bio-Plex Manager™ software versions 4.0 and 5.0 (Bio-Rad Laboratories Inc.)

2.10 Perfusion and immunohistochemistry

Two perfusion protocols were used for detection of pSTAT3 and cFos immunoreactivity in mouse brain tissue.

2.10.1 Perfusion protocol for pSTAT3

Mice were anesthetized with pentobarbital intraperitoneally, and perfused intracardially (10ml/min) with 0.9% NaCl for 90 seconds, followed by ice-cold 2% paraformaldehyde (Sigma-Aldrich, pH 7.2) in potassium-phosphate-buffered saline (KPBS) for 120 seconds via the ascending aorta. Following perfusion, the brain was removed by dissection, post-fixed in 2% paraformaldehyde solution for 2 hours, and cryoprotected in 20% sucrose KPBS solution for 36-48 hours at 4°C.

2.10.2 Perfusion protocol for cFos

Mice were anesthetized with pentobarbital intraperitoneally, and perfused intracardially (10ml/min) with ice-cold 0.1M phosphate buffer (PB, Sigma-Aldrich, pH 7.2) for 90 seconds, followed by ice-cold 4% paraformaldehyde in 0.1M PB (pH 7.2) for 120 seconds via the ascending aorta. Following perfusion, the brain was removed by dissection, and post-fixed in 4% paraformaldehyde solution for 2 hours, and cryoprotected in 20% sucrose PB solution for 36-48 hours at 4°C.

2.10.3 Brain tissue processing

The brains were frozen in chilled Hexane (Fluka Chemie GmbH, Buchs SG, CH) on dry ice, and stored at -20°C until sectioned coronally using a cryostat (Leica CM3050S, Nussloch, Germany). Twenty-micrometer sections collected from the forebrain (containing VMH and ARC) and the hindbrain (containing AP and NTS; according to Paxinos and Watson, 2007) were thawed mounted onto adhesion glass slides (Superfrost Plus, Gerhard Menzel GmbH, Braunschweig, Germany).

2.10.4 Protocol for pSTAT3-immunostaining

Frozen slides were first brought to room temperature and then rinsed in 0.02M KPBS for 10 minutes. Slides were subjected to the following antigen-retrieval and blocking steps, with rinsing in 0.02M KPBS between each step: 0.3% NaOH + 0.3% H_2O_2 in KPBS for 20 minutes, 0.3% glycine in KPBS for 10 minutes, 0.03% sodium dodecyl sulfate (SDS) in KPBS for 10 minutes, and blocked in 4% natural donkey serum (NDS, Jackson ImmunoResearch Inc., Newmarket, Suffolk, UK) with 0.4% Triton and 1% bovine serum albumin (BSA) in KPBS for 20 minutes. The primary antibody (1:500, Phospho-Stat3 (Tyr705) (D3A7) XP™ Rabbit mAb, Cell Signaling Technology Inc., Denver MA, US) was added to the blocking solution with 1% NDS (Jackson ImmunoResearch Inc.), 0.4% Triton and 1% BSA in KPBS and incubated for 48 hours at 4°C. Then, sections were washed in KPBS for 45 minutes, incubated with donkey anti-Rabbit Alexa-555 IgG (H+L) (1:100; Invitrogen AG, Basel, CH) in

1% NDS with 0.3% Triton in KPBS for 2 hours at room temperature. As a final step, slides were rinsed in 0.02M KPBS for 45 minutes before being covered-slipped with Citifluor™ (Glycerol/PBS solution AF1; Citifluor Ltd., London, UK).

2.10.5 Protocol for cFos-immunostaining

Frozen slides were first brought to room temperature and then rinsed in phosphate-buffered saline (PBS; pH 7.4) containing 0.1% phosphate-buffered saline with Triton X-100 (PBST). Slides were first incubated in NDS (1.5% in 0.3% PBST; Jackson ImmunoResearch Inc.) for 2 hours, followed by a 48-hour incubation in polyclonal rabbit anti-cFos (1:5,000 in 0.3% PBST; AB-5, Oncogene) at 4°C. Following 50 minutes of rinsing in 0.1% PBST, sections were incubated in biotinylated donkey anti-rabbit IgG (1:400 in 0.3% PBST; Jackson ImmunoResearch Inc.) for 90 minutes at room temperature. Slides were rinsed with 0.1% PBST for 30 minutes, and then incubated for 90 minutes in avidin-biotin-peroxidase complex (1:100 in 0.3% PBST, Vectastain ABC kit, LINARIS GmbH, Dossenheim, Germany). Sections were then rinsed once with 0.1% PBST for 10 minutes and once in Tris(hydroxymethyl)aminomethane-hydrochlorid acid (Tris-HCl) for 10 minutes before being incubated in 3,3'-Diaminobenzidine Tetrahydrochlorid (DAB, Sigma-Aldrich) solution for 8 minutes to visualize cFos-positive cells. The DAB-solution was made in Tris-HCl (0.04% DAB, 0.008% H_2O_2). After rinsing the slides with Tris-HCl for 5 minutes and 0.1% PBST for 5 minutes, they were dehydrated in graded alcohol (50%, 75%, 95%, 100%) and immersed in xylol for 5 minutes each before being cover-slipped with Entellan (Merck KGaA, Darmstadt, Germany).

2.11 Description of individual experiments

2.11.1 Experiment 1: Metabolic phenotyping of H1rKO mice

2.11.1.1 Experiment 1a

In a first study, C57BL/6J WT mice (BW: 32 ± 4g; n=6) and H1rKO mice (BW: 37 ± 4g; n=8) at an average age of 6 months were used for blood sampling. Plasma samples were used to perform hormone analysis for leptin, insulin and amylin. Following blood sampling and sacrifice, the carcasses were used for CT scan to assess body composition by estimating the amount of intraabdominal and subcutaneous body fat.

2.11.1.2 Experiment 1b

In a second study, a separate group of WT mice (BW: 28 ± 2g; n=8) and H1rKO mice (BW: 31 ± 3g; n=8) at an average age of 5 months were housed in metabolic cages to perform metabolic baseline measurements including EE, RQ, physical activity, 24-hour food intake and food intake subsequent to 6-hour fasting. After an adaptation period of 5 days in the metabolic cages, the 24-hour data were recorded by the AccuScan system over three days; results are presented as the average of the three days. As an additional experiment to determine the feeding behavior of WT and H1rKO mice after a short period of fasting, access to the food hoppers was blocked for 6 hours from 0700h until dark onset (1300h). Food intake was measured automatically from dark onset and data for 1, 2 and 4-hour food intake were analyzed using the Integra program.

2.11.2 Experiment 2: Effects of amylin on food intake and cFos induction in H1rKO mice

2.11.2.1 Experiment 2a in *ad libitum* fed mice

WT mice (BW: 28 ± 2g; n=12) and H1rKO mice (BW: 33 ± 4g; n=11) at an average age of 5 months were used to investigate the role of H1 receptors in amylin's anorectic effect by assessing food intake following administration of different amylin doses.

Mice were individually housed in hanging wire mesh cages for 2 weeks prior to the start of the experiment to allow for adaptation to the new, individual housing conditions. During this time, mice were taken out of the cages periodically for body weight measurement and adaptation to the restraint position necessary for intraperitoneal injections. Mice had free access to water and chow, except at the day of experiment for about 15-30 minutes during injections.

The experiment was conducted in a crossover manner consisting of 4 trials in which each mouse was exposed to each treatment dose. On experimental days, food was removed from the cages 15-30 minutes before the start of each trial. Mice were injected with saline or amylin (5, 20 or 50μg/kg; i.p.) just before dark onset (1300h) with an injection volume of 0.1ml/10g bodyweight, following which food was immediately returned to the cage. Food intake was measured by manually weighing the pellets in the food hoppers, trying to avoid contamination by urine and feces. Food intake was measured 1, 2 and 4 hours after injection.

Two days after the last feeding trial, non-fasted WT mice (n=12) and H1rKO mice (n=6) of the same cohort were injected with either saline or amylin (50μg/kg; i.p.) at dark onset (1300h) for the terminal experiment. Just after the injections, food was taken out of the cages and mice were anesthetized and perfused two hours later. In this particular case, the pSTAT3 perfusion protocol had been used for detection of cFos; this did not compromise the detection of cFos immunoreactivity. cFos-positive cells were counted in the AP under light microscopy.

2.11.2.2 Experiment 2b in 6-hour fasted mice

Because a significant effect of amylin on food intake was not observed in H1rKO mice at the doses administered in Experiment 2a, an additional experiment was conducted to assess the effect of an additional, higher amylin dose (200µg/kg; i.p.) in WT (BW: 28 ± 2g; n=4) and H1rKO mice (BW: 30 ± 1g; n=4) which had an average age of 5½ months. These mice were kept in metabolic cages and were adapted to the new environment conditions for 7 days before the feeding trials started. Similar to Experiment 2a, the experiment was conducted in a crossover manner consisting of five trials.

On experimental days, the animals were food restricted for the last 6 hours of the light phase (0700-1300h). Immediately prior to dark onset, mice were injected with either saline or amylin at a dose of 5, 20, 50 or 200µg/kg (i.p.) with an injection volume of 0.1ml/10g BW. Following completion of all injections, food was immediately returned. Food intake was recorded automatically by the AccuScan system, and 1, 2 and 4-hour food intake was analyzed with the Integra program.

For the terminal experiment, four WT mice and four H1rKO mice were fasted for 6 hours prior to receiving an injection of either saline or amylin (50µg/kg; i.p.) at dark onset (1300h), and then anesthetized and perfused two hours later. Mice were perfused using the cFos perfusion protocol and cFos-positive cells were counted in the AP under light microscopy.

2.11.3 Experiment 3: Effects of leptin on food intake and pSTAT3 induction in H1rKO mice

The mice used in Experiment 1b for basic metabolic measurements were used in this experiment. Wild type mice (BW: 28 ± 2g; n=8) and H1rKO mice (BW: 30 ± 4g; n=8)) at an average age of 5 months were used to investigate leptin's effect on eating by assessing food intake following administration of various leptin doses. The experiment was performed in a cross over manner consisting of five trials.

The mice remained housed in the metabolic cages following Experiment 1b. On experimental days, access to food was blocked in the middle of the light phase at 0700h, and the mice were injected with either saline or leptin (500μg/kg, 1, 5 or 10mg/kg; i.p.) 2 hours prior to dark onset (1100h). At dark onset (1300h), mice were given access to food and food intake was measured for 1, 2 and 4 hours automatically by the AccuScan system. Data were analyzed using the Integra program.

In a separate experiment using a different cohort of mice, non-fasted WT mice (BW: 27 ± 2g; n=5) and H1rKO mice (BW: 32 ± 2g; n=6) were injected with either saline or leptin (1.3mg/kg; i.p.) at dark onset (1300h), and then anesthetized and perfused 45 minutes later. The pSTAT3 perfusion protocol was used for detection of pSTAT3-positive cells, which were counted in the VMH and ARC from photomicrographs obtained under fluorescent microscopy.

2.11.4 Experiment 4: Effects of amylin and leptin co-treatment on H1rKO mice

The same animals as in Experiments 1b and 3 were used to investigate the effect of amylin and leptin co-treatment on food intake. The experiment was assessed in a crossover manner across four trials testing four different treatment groups: Saline-saline, saline-amylin, saline-leptin and amylin-leptin. Wild type mice (BW: 28 ± 2g; n=8) and H1rKO mice (BW: 30 ± 4g; n=8) at approximately 5½ months of age were fasted for the last 6 hours of the light phase (0700-1300h). Two hours prior to dark onset (1100h) the mice were injected with saline or leptin (1mg/kg; i.p.). Just before dark onset (1300h), the animals were injected with either saline or amylin (50μg/kg; i.p.), and access to food was given immediately following the completion of injections (see Figure 2.2). Food intake was recorded automatically by the AccuScan system and data of 1, 2 and 4-hour-food intake were analyzed using the Integra program.

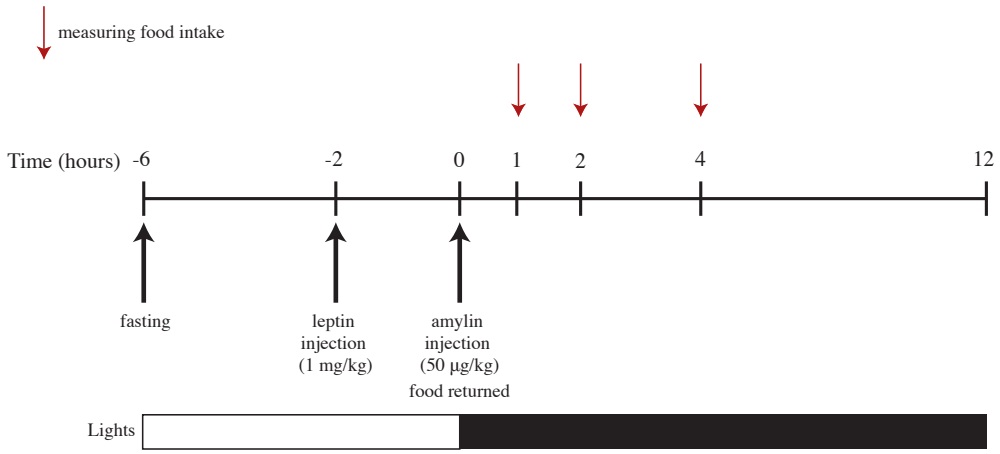

Figure 2.2: Timeline of Experiment 4

2.12 Statistical analysis

All data are expressed as mean ± SEM. In experiments comparing independent treatment groups, a 1-factor ANOVA was used to detect statistically significant differences between the control saline and the different dose groups, with Bonferroni's Multiple Comparison Test used to determine differences between individual groups. When more than two factors were compared (genotype x dose), data were analyzed using a 2-factor ANOVA, with Bonferroni's post-hoc test used to determine differences between individual groups. In some cases, the Mann-Whitney U test was used for non-parametric comparison of independent groups. A p-value <0.05 was considered to be statistically significant. Statistical analyses were performed using the GraphPad Prism (version 5.0a, San Diego, CA, USA).

2.13 Supplemental methods

2.13.1 Instruction for mouse handling

When laboratory mice are handled, they typically display defensive behaviors such as biting and fleeing from the experimenter; this is very stressful for laboratory animals and the experimenter. The goal of the following handling procedure was to decrease

or even stop these behaviors, which results in less stress for both animals and investigator. Other studies have shown that animal handling improves experimental outcome (Ryabinin et al., 1999); this has been previously established in our laboratory by Becskei (Becskei et al., 2009).

Based on personal communication with Dr. Gustavo Pacheco Lopez (Physiology and Behavior Laboratory, ETH Zurich), who applies the so-called meatball technique in mice intended for brain cannulation, this specific handling technique was extended by a self-invented desensitization program. The handling procedure is based on the desensitization principle established by Joseph Wolpe (Wolpe, 1961, 1963, 1969).

The mice were handled daily. The investigator begins by wearing thick gloves to avoid being bitten. One hand firmly holds the mouse's tail to secure the mouse on a cage lid grid. With the other hand, the investigator carefully strokes the animal's fur, which will trigger the mouse to try to bite in defense. A pencil tip can also be used to stroke the mouse to provide more protection in the first days of handling. Contact with the animal is maintained as long as it continues biting, but only limited pressure should be applied on its body. As soon as the mouse ceases to bite, the finger or pencil should be removed. The mouse will learn quickly that as long as it is biting, the object is not going to be removed. The initial days require much patience. Upon successfully learning not to bite, the mouse can be returned to the cage, and practice will continue next day again.

With daily handling the mice will stop biting, and after approximately one week the mice are noticeably calmer. It is observable that the mice urinate and defecate less, and progressively, cease to show this sign of stress altogether. The intensity of touch can be increased when the mice tolerate gentle touches. The more time that is given to them to accept handling, the better they learn not to be afraid. As a next step, e.g. when i.p. injections are practiced, the mice will start to be stressed again, because of a new situation. Again, it is possible for them to get used to being held in the back restraint position and tolerate the pain. Therefore it is important to proceed step by step and not to overload the animals.

For longer experiments, where it is known that the animal will stay under the researcher's care for several weeks, it may help to spend more time with the animals. For instance, the first contact with the mice can be to simply place a gloved hand into the cage without doing anything additional. The animals will then begin to investigate and smell the fingers, and eventually will try to climb up the investigator's arm. At this point when the mice are used to the hand sitting in the cage, it is much easier to retrieve them from the cage.

Based on my personal experience with handling mice in this manner, I conclude that mice are quite intelligent and learn quickly. The transformation of the mouse behavior from the start to the end of an experiment was truly amazing. I highly recommend taking the additional time to handle mice in this way. It really benefits the mice and the experiment.

3 Results

3.1 Experiment 1: What are the characteristics of H1rKO mice?

3.1.1 Body weight and fat distribution

In the first experiment, we sought to further phenotype the H1rKO mice. Body weight data at weaning were not available. The mice were on average 19 weeks (4½ months) old when recruited for this experiment. They had free access to standard chow and water, and were group housed with 2-5 mice per Makrolon cage. Figure 3.1A shows that the body weight of H1rKO mice was significantly higher ($p<0.001$) than the WT mice for the entire observation time leading up to the final sampling day (Fig. 3.1B). Blood sampling was performed and body composition was analyzed at about 6 months of age.

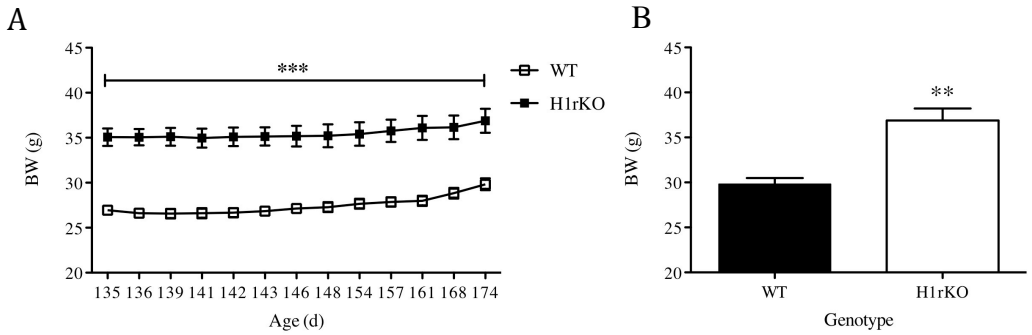

Figure 3.1: Body weight in WT mice (n=6) and H1rKO (n=8) over 40 days (A) and on the final sampling day (B). All data are expressed as mean ±SEM, symbols denote significant differences between the two genotypes; **p<0.01, ***p<0.001

To assess body composition of the two genotypes, body fat was measured using CT scan. The amount of adipose tissue located between the diaphragm and the hip joints was measured to compare subcutaneous and intraabdominal body fat content in both genotypes. Figure 3.2 shows that the H1rKO mice had significantly more subcutaneous ($p<0.05$) and intraabdominal ($p<0.05$) fat than WT mice, which was consistent with the higher body weight in H1rKO mice. To account for the higher

body weight in H1rKO mice, if fat mass was normalized to total body weight, H1rKO mice still exhibited a higher ratio of fat mass to total weight compared to WT controls (data not shown), confirming increased adiposity.

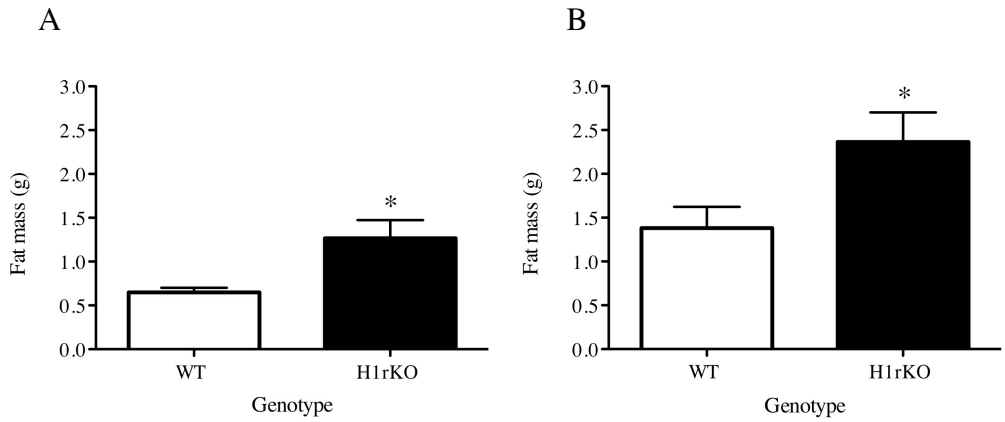

Figure 3.2: Subcutaneous (A) and intraabdominal (B) fat mass in WT (n=6) and H1rKO (n=8) mice. All data are expressed as mean ±SEM. The symbol denotes significant differences between the two genotypes; *p<0.05

3.1.2 Circulating hormones

Circulating hormones were measured to characterize the baseline level of satiation and adiposity hormones in H1rKO mice compared to WT mice after a fasting period of 4 hours. Figure 3.3 shows that H1rKO had significantly higher leptin levels ($p<0.05$) than WT and demonstrated a tendency for higher levels of amylin and insulin. These results correspond with elevated body weight and body adiposity observed in the H1rKO mice.

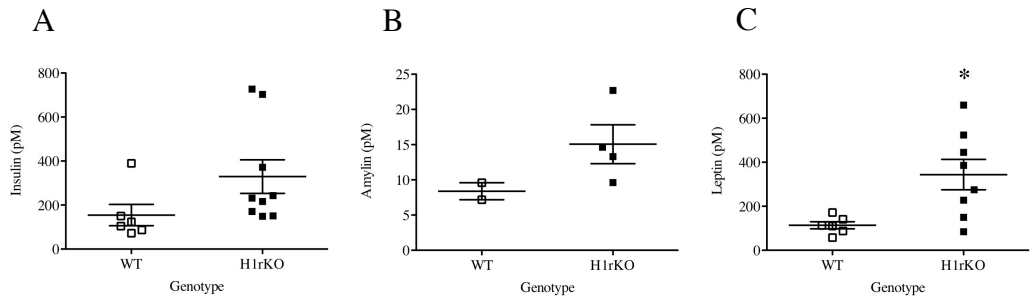

Figure 3.3: Fasting baseline insulin (A), amylin (B) and leptin (C) blood levels in WT (n=6) and H1rKO (n=8) mice at an age of 6 months. Individual data are shown and all data are also expressed as mean ±SEM. The symbol denotes a significant difference between the two genotypes; *p<0.05

3.1.3 Energy expenditure and activity

To assess metabolic parameters in H1rKO mice compared to WT mice, energy expenditure (EE), respiratory quotient (RQ), food intake (FI) and physical activity (PA) were measured over 3 days using open circuit indirect calorimetry. Average data over the three days are presented. As shown in Figure 3.4A, the average EE was not significantly different between the light and dark phase, nor was there a clear genotype effect. However, the H1rKO showed a tendency toward lower EE. Two-way ANOVA detected an overall light phase effect ($p<0.01$) but no genotype effect for RQ (Fig. 3.4B). Follow-up analysis in both genotypes showed a tendency to decreased respiratory quotient during the light phase, but the effect was not significant.

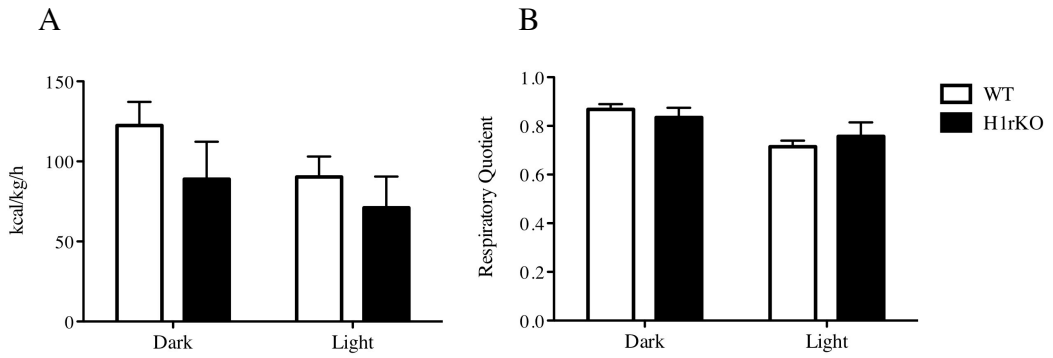

Figure 3.4: Energy expenditure (A) and average respiratory quotient (B) in WT (n=8) and H1rKO (n=8) mice during dark and light phase. All data are expressed as mean ±SEM

Figure 3.5 shows the physical activity in WT and H1rKO mice during the dark and light cycle. There was no genotype effect but overall markedly lower physical activity during the light phase ($p<0.01$).

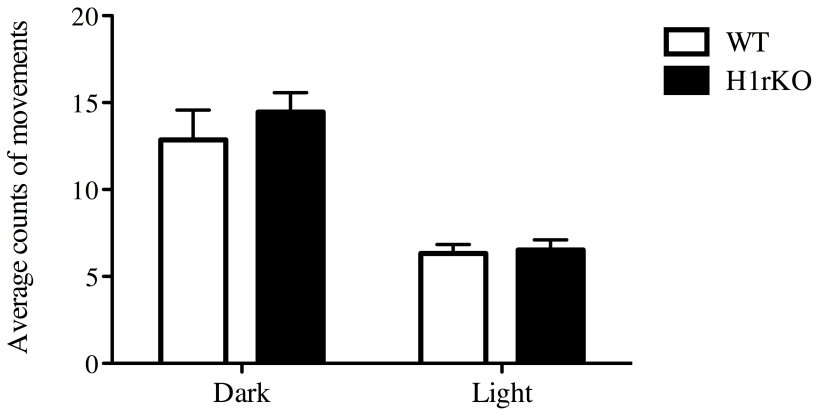

Figure 3.5: Physical activity in WT (n=8) and H1rKO (n=8) mice during dark and light phase. All data are expressed as mean ±SEM

3.1.4 24-hour food intake in WT and H1rKO mice

To investigate the baseline feeding behavior in H1rKO mice, cumulative 24-hour food intake was measured over three days, and the average of these three days was analyzed. Two-way ANOVA did not detect a genotype effect but a very clear overall light phase effect ($p<0.01$; Fig. 3.6).

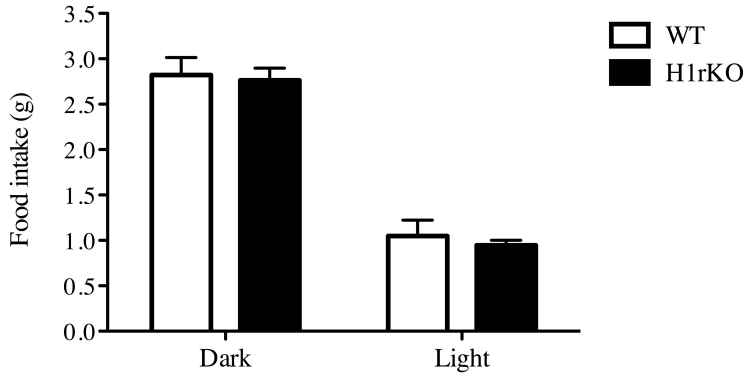

Figure 3.6: Average cumulative FI during the dark and light phases measured over 3 days in WT (n=8) and H1rKO (n=8) mice. All data are expressed as mean ±SEM

Analysis of hourly food intake (Fig. 3.7) again showed no genotype effect but a clear effect of time ($p<0.001$). The H1rKO mice showed a tendency toward higher FI compared to WT mice in the first three hours after the onset of dark.

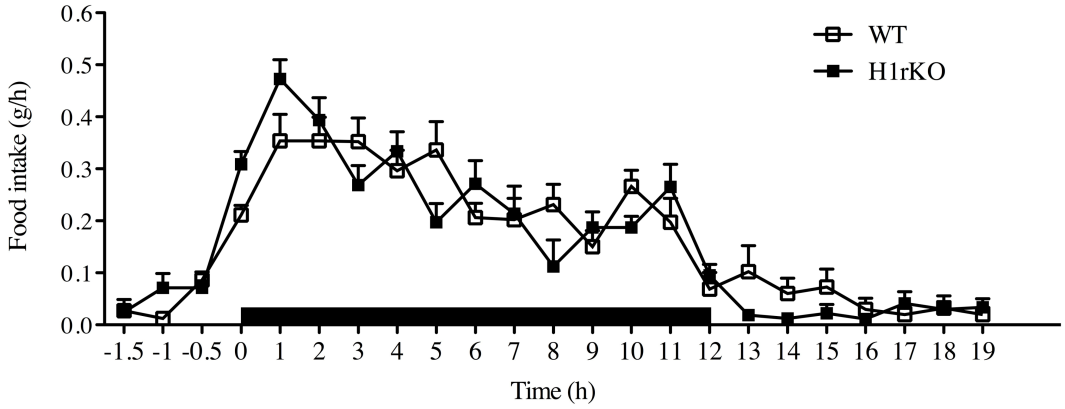

Figure 3.7: Average hourly FI during 24 hours in WT (n=8) and H1rKO (n=8) mice. All data are expressed as mean ±SEM. The black bar indicates the dark phase; time point 0=1300h; 12=0100h

3.1.5 Fasting induced food intake

To test whether the tendency of increased food intake in H1rKO in the first hours after dark onset can be augmented, the animals were fasted for 6 hours prior to dark

onset in the next experiment. The same cohort of 5 month old animals was used. H1rKO mice showed significantly higher food intake in the first few hours after the fasting period compared to WT mice. Two-way ANOVA detected a main effect of genotype ($p<0.001$) and of time ($p<0.001$) for the first four hours. However, the genotype effect was no longer present after 12 hours (Fig. 3.8).

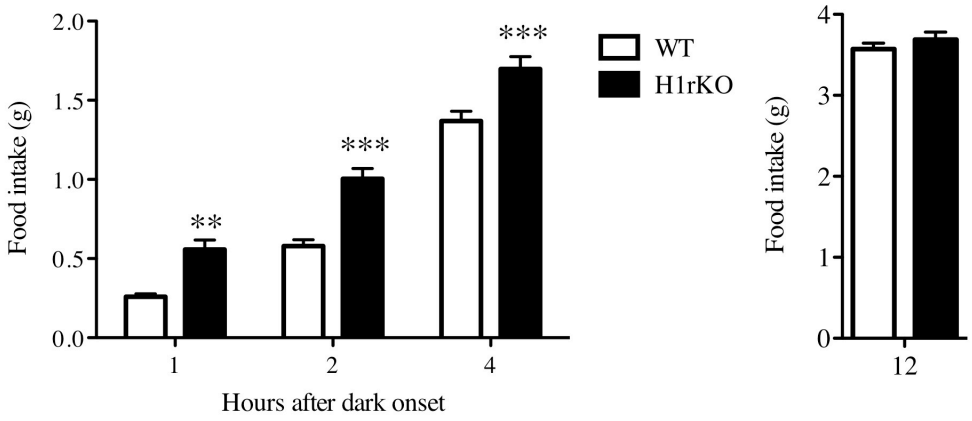

Figure 3.8: 6 hour-fasting-induced cumulative FI in WT (n=8) and H1rKO (n=8) 1, 2, and 4 hours (A) and 12 hours (B) after refeeding. All data are expressed as mean ±SEM. Symbols denote significant differences between the two genotypes; **p<0.01, ***p<0.001

3.2 Do mice lacking H1 receptors respond to anorectic doses of amylin?

The aim of this experiment was to investigate whether various doses of amylin (5, 20, 50 and 200μg/kg) reduce eating in H1rKO mice to the same extent as in WT mice.

3.2.1 1-hour food intake after amylin administration

Figures 3.9 and 3.10 show the cumulative FI in the first hour after amylin administration (5, 20 and 50μg/kg) in WT and H1rKO mice at an average age of 5 months. Two-way ANOVA detected significant main effects of both genotype ($p<0.05$) and amylin treatment ($p<0.05$), and a significant difference between WT and KO mice observed following the 20μg/kg dose (Fig. 3.9).

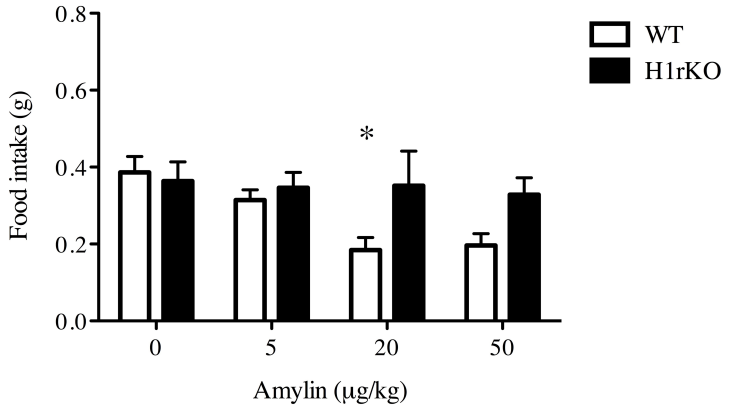

Figure 3.9: Cumulative food intake 1 hour after saline or amylin (5, 20, 50µg/kg) administration in WT (n=12) and H1rKO (n=6) mice. All data are expressed as mean ±SEM. Symbol denotes significant difference between the two genotypes; *p<0.05. Two-way ANOVA detected a significant genotype effect (p<0.05) and an overall treatment effect (p<0.05)

Plotted in a different way, Figure 3.10 illustrates the effect of amylin on food intake, as compared to saline, one hour after administration. The WT mice showed significantly reduced food intake compared to saline-treated mice ($p < 0.001$; Fig. 3.10A), with significant individual differences at 20 and 50µg/kg between amylin and saline. This was not the case for H1rKO mice, which did not respond to any amylin dose tested (Fig. 3.10B).

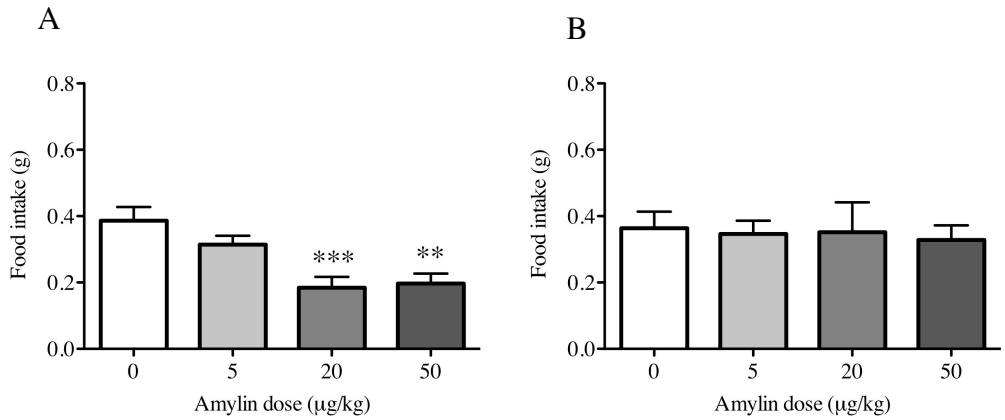

Figure 3.10: Food intake after 1 hour in WT (A) and H1rKO mice (B). Symbols denote significant differences between the amylin-treated and the control saline groups; **p<0.01, ***p<0.001. One-way ANOVA detected a treatment effect in WT mice (p<0.001), but not in H1rKO mice

While WT mice demonstrated significant decreases in food intake in the first hour following amylin treatment, the effect of amylin was no longer significant 2 or 4 hours after treatment, and the effect was completely abolished 12 hours after amylin administration. Similar to the data from the first hour after amylin treatment, H1rKO mice did not show any effect of amylin on food intake at later time points (data not shown).

Because we did not observe an amylin-induced suppression in food intake with the three doses tested, the feeding trials were repeated in the metabolic cages with a second cohort of animals, in which we also assessed the effect of an additional, higher dose of amylin (200μg/kg) on food intake in WT and H1rKO mice. The results for 5, 20 and 50μg/kg amylin administration were comparable with the previous trial (data not shown). Unlike the H1rKO mice, the WT showed a significant reduction in 1-hour FI after amylin (20, 50 and 200μg/kg) compared to the WT saline control group.

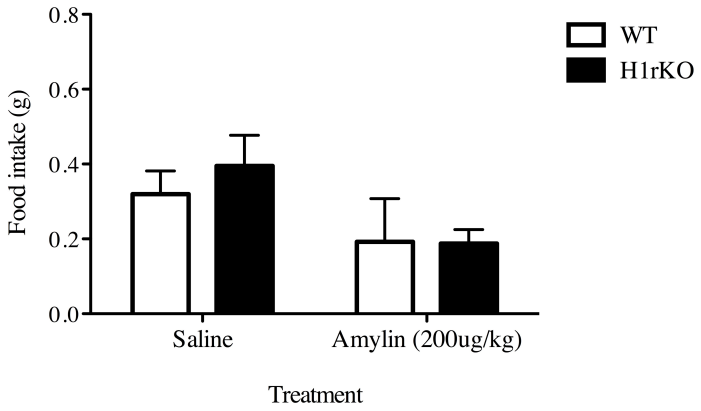

Figure 3.11: Cumulative 1 hour food intake after saline or amylin (200µg/kg) administration in WT (n=4) and H1rKO (n=4) mice. All data are expressed as mean ±SEM

With the highest dose of 200µg/kg, a main effect of amylin was observed in both WT and H1rKO mice ($p<0.05$), and no difference between genotypes was detected (Fig. 3.11). Thus, compared to the WT mice, the H1rKO mice were generally less responsive to amylin administration unless the high dose of 200µg/kg was given.

3.2.2 cFos activity in the area postrema

Although food intake was not suppressed by amylin in H1rKO mice up to a dose of 50µg/kg, the aim of the final experiment was to test if amylin may still induce cFos expression in the AP. The dose of amylin chosen to potentially induce a cFos response was the highest dose that had no effect on eating in the H1rKO mice at any time point. Thus, both WT and KO mice were injected with either saline or amylin (50µg/kg) at dark onset and were sacrificed 2 hours later. Due to brain sectioning problems, not all mice could be used for analyzing.

Two-way ANOVA detected a genotype effect ($p<0.05$) and a treatment effect ($p<0.001$; Fig. 3.12 and 3.13). While both genotypes showed elevated cFos activity after amylin administration, the H1rKO mice expressed a significantly higher number of cFos positive cells compared to the WT mice, despite showing no anorectic

response after amylin. Representative photomicrographs of the AP are shown in Figure 3.13.

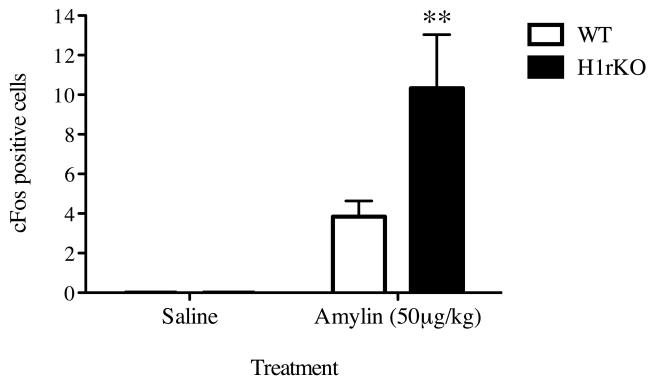

Figure 3.12: Number of cFos positive cells in the AP of WT (n=4) and H1rKO (n=3) mice 2 hours after saline or amylin (50µg/kg) injection. All data are expressed as mean ±SEM. Symbol denotes significant differences between the two genotypes; **p<0.01

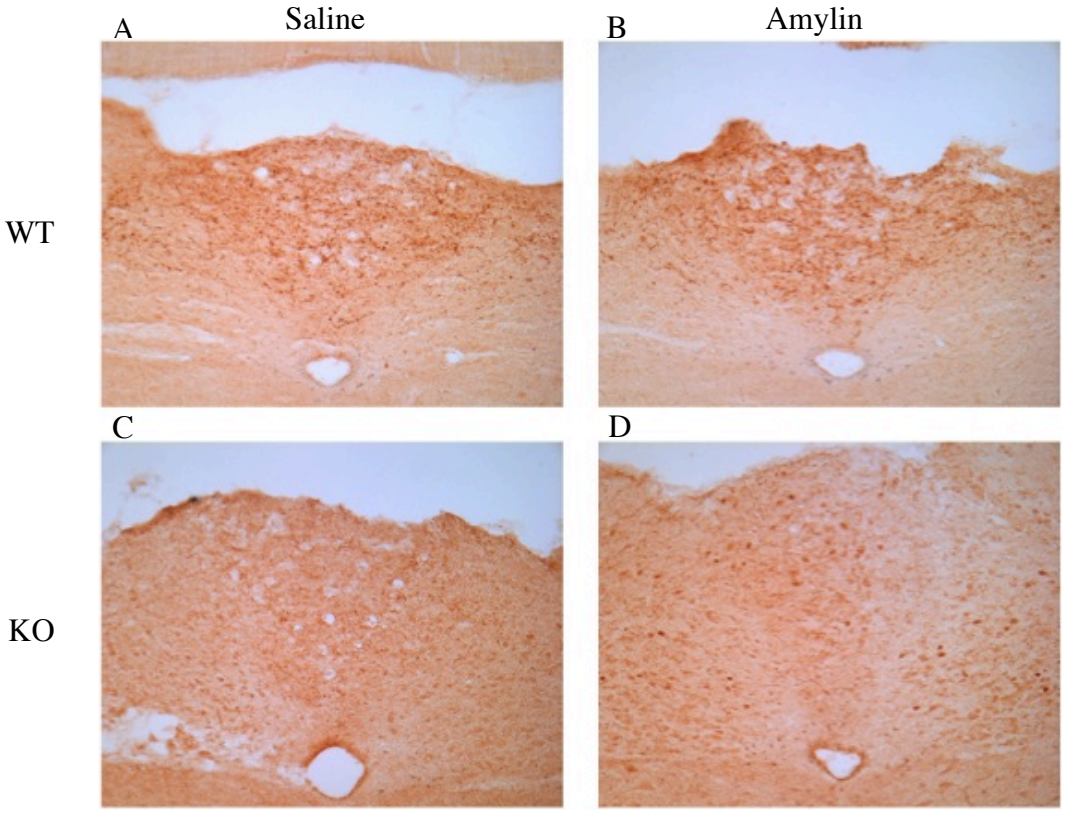

Figure 3.13: Representative photomicrographs of cFos positive cells in the area postrema after saline (A, C) or amylin administration (50µ/kg; B, D) in WT (A, B) and H1rKO (C, D) mice

3.3 Do mice lacking H1 receptors respond to anorectic doses of leptin?

The aim of this experiment was to investigate whether various doses of leptin (500µg/kg, 1, 5 and 10mg/kg) reduce eating in H1rKO mice to the same extent as in WT mice.

3.3.1 Food intake after leptin administration

Figure 3.14 shows cumulative food intake 1, 2 and 4 hours after dark onset, i.e. 3, 4 and 6 hours after saline or leptin administration, in WT and H1rKO. The data show that none of the leptin doses significantly reduced food intake in H1rKO mice in the first two hours with food access, and that H1rKO mice were less responsive to higher doses of leptin (5 and 10mg/kg) at 4 hours after dark onset, compared to the WT controls. In contrast, the WT mice showed reduced food intake already in the first

hour of food access following the 5mg/kg dose, and responded to all doses of leptin at either the 2- or 4-hour time point.

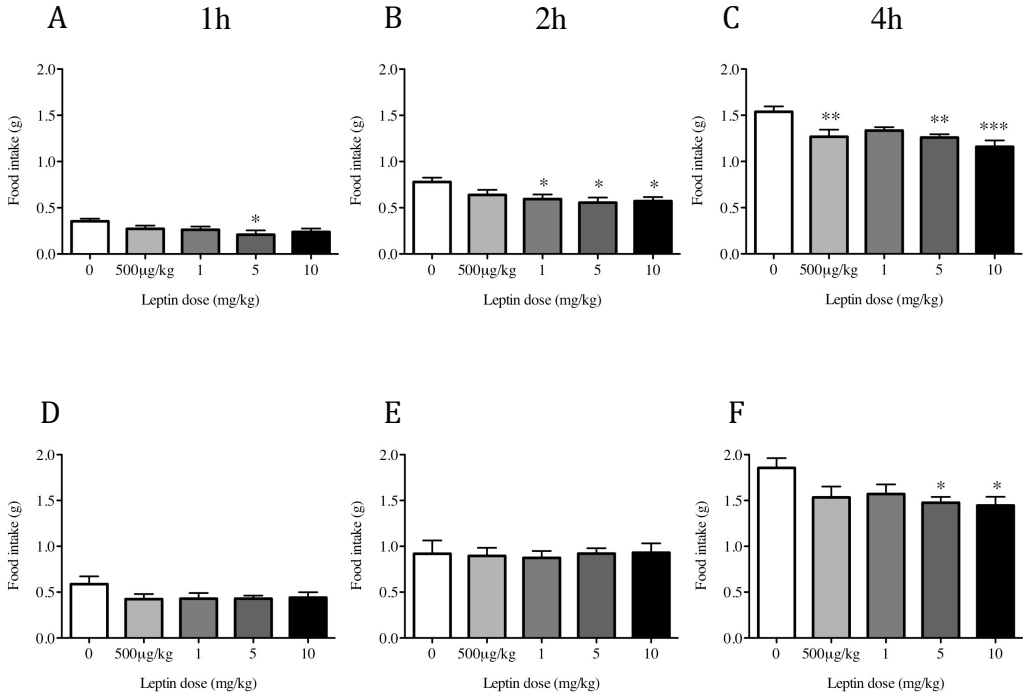

Figure 3.14: Cumulative food intake 1 hour (A, D), 2 hours (B, E) and 4 hours (C, F) after dark onset in WT mice (n=8; A-C) and H1rKO mice (n=8; D-F). Animals were injected with either saline or leptin (500µg/kg, 1, 5 or 10mg/kg) 2 hours prior to dark onset and access to food. All data are expressed as mean ±SEM. Symbols denote significant differences between the leptin treated and the control saline group; *p<0.05, **p<0.01, ***p<0.001

3.3.2 pSTAT3 activity in the hypothalamus

In the final experiment the aim was to test if leptin induces pSTAT3 activity in two nuclei of the hypothalamus (VMH and ARC), which are two main sites of leptin action. Mice were injected with either saline or leptin (1.3mg/kg), sacrificed 45 minutes later, and the brains were processed to detect pSTAT3-positive neurons (Fig. 3.15).

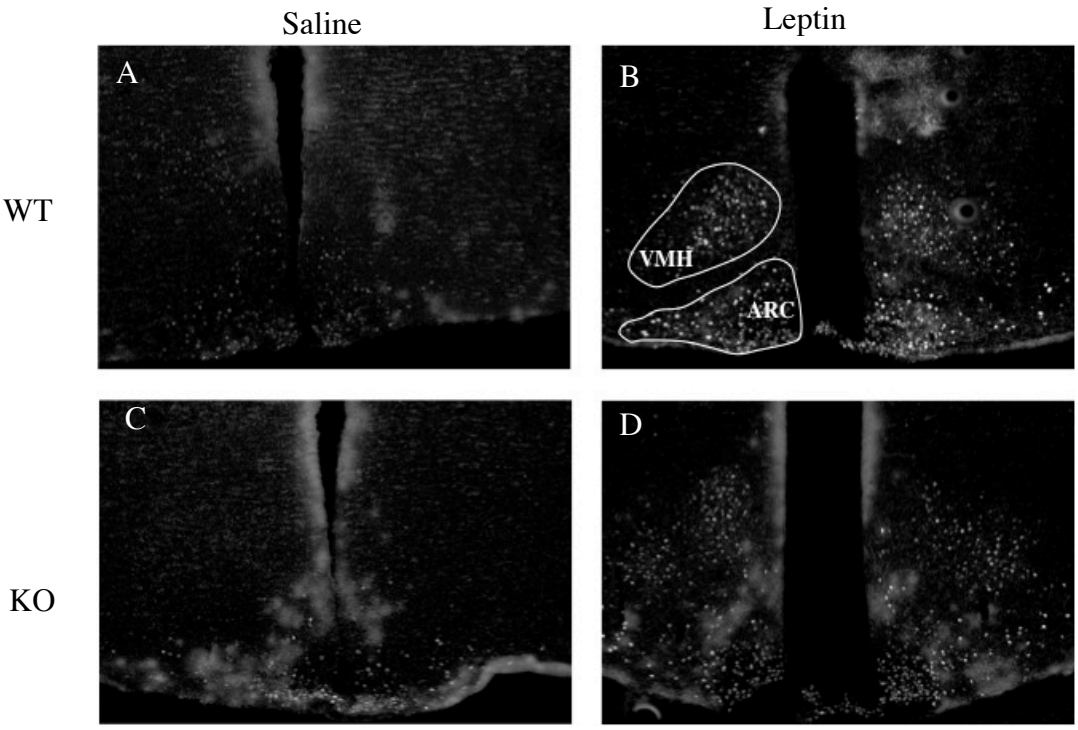

Figure 3.15: Representative photomicrographs of pSTAT3-positive cells within the arcuate nucleus (ARC) and the ventromedial nucleus of the hypothalamus (VMH) after saline (A, C) or leptin (1.3mg/kg; B, D) administration in WT (A, B) and H1rKO mice (C, D)

Two-way ANOVA did not detect a genotype effect in either brain area, but there was a strong main effect of leptin in both the ARC ($p<0.001$) and the VMH ($p<0.001$). T-test analysis showed significance in the WT animals comparing the effect of saline versus leptin in the ARC ($p<0.001$) and the VMH ($p<0.01$). In H1rKO mice the t-test showed significance only in the VMH ($p<0.01$) but a clear tendency in the ARC when comparing saline versus leptin treatment (Fig. 3.16). These data therefore demonstrated leptin receptor responsiveness in H1rKO mice, despite the lack of a leptin effect on food intake at a similar dose (1mg/kg).

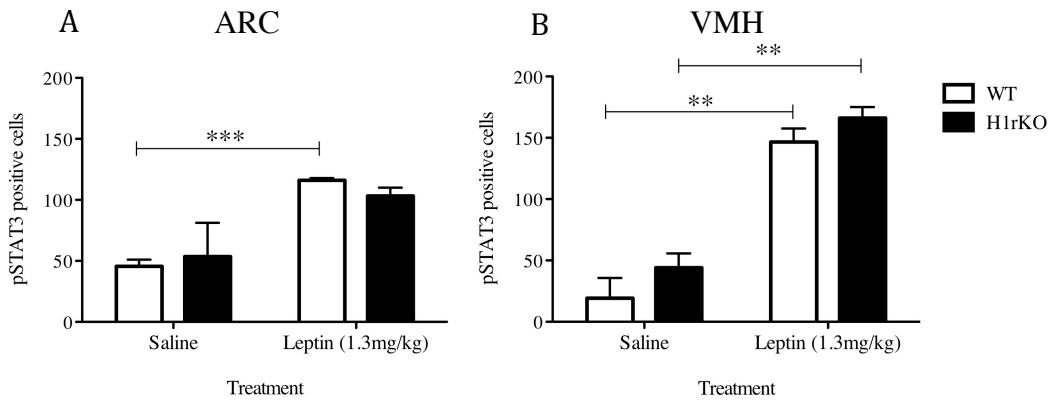

Figure 3.16: Quantification of the number of pSTAT3-positive cells in the ARC (A) and the VMH (B) in WT (n=5) and H1rKO mice (n=6) 45 minutes after either saline or leptin (1.3mg/kg) injection. All data are expressed as mean ±SEM. Symbols denote significant differences between treatment groups within the same genotype; **p<0.01, ***p<0.001

3.4 Do mice lacking H1 receptors respond to a combined amylin – leptin administration?

To investigate the interaction of amylin and leptin, a combined treatment was assessed using the same cohort of animals as in experiments of section 3.3 (Experiment 4). The animals were fasted for 6 hours. Saline or leptin (1mg/kg, i.p.) was injected 2 hours prior to dark onset; at dark onset, saline or amylin (50µg/kg, i.p.) was injected just before access to food was provided. Figure 3.17 shows cumulative food intake 1 hour and 2 hours after dark onset in WT and H1rKO mice.

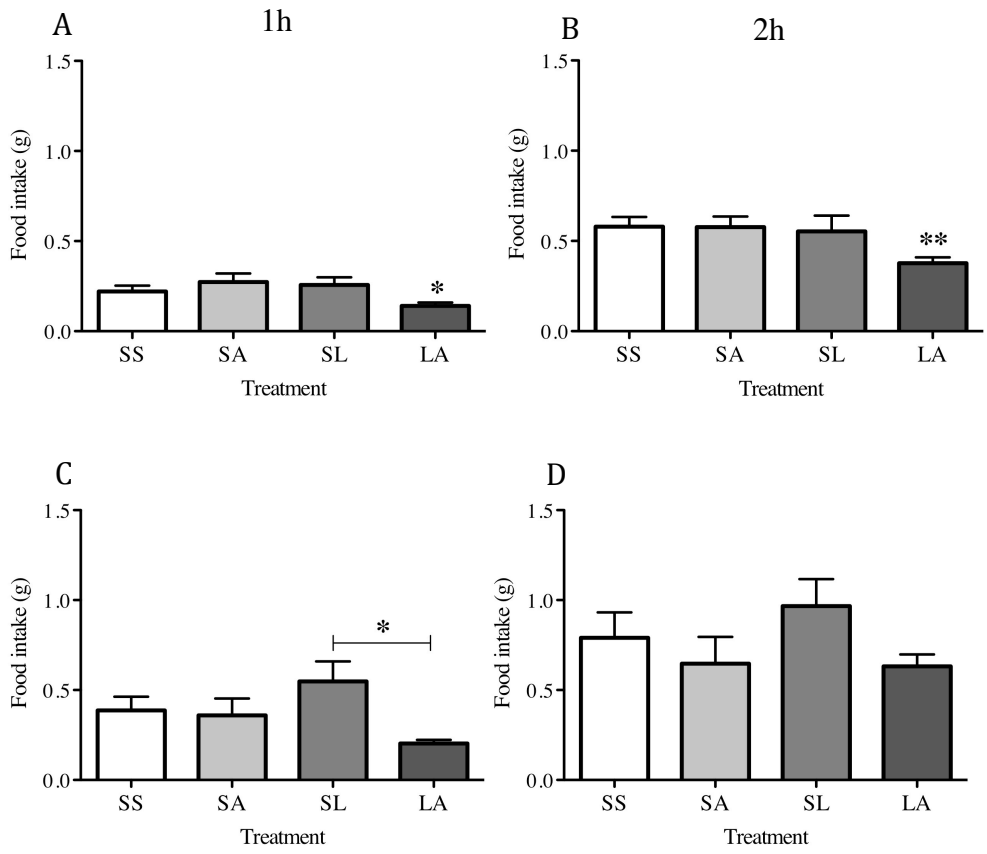

Figure 3.17: Cumulative food intake 1 hour (A, C) and 2 hours (B, D) after dark onset in WT mice (n=8; A, B) and H1rKO mice (n=8; C, D), injected with saline (S) or leptin (L; 1mg/kg) two hours prior to dark onset and with saline (S) or amylin (A; 50µg/kg) at dark onset. All data are expressed as mean ±SEM. Symbols denote significant differences between the treatment groups and the control saline-saline group; *p<0.05, **p<0.01

Co-treatment of amylin and leptin in WT mice resulted in a significant decrease in 1 and 2 hour-food intake, compared to saline treatment, (1 hour: $p<0.05$; Fig. 3.17A; 2 hours: $p<0.01$; Fig. 3.17B). WT mice did not show reduced food intake after either amylin or leptin treatment alone, i.e. the outcome was different compared to the single-treatment experiments using the same doses of amylin and leptin (experiments of section 3.2 and 3.3). Unlike the WT mice, the H1rKO mice did not show decreased food intake after any treatment combination compared to controls (Fig. 3.17C and D). Notably, no responsiveness to the combined amylin-leptin treatment was detected in H1rKO mice.

4 Discussion

The aim of the study was to test the hypothesis that the histaminergic system mediates the interaction between amylin and leptin to control food intake and body weight. Using mice deficient in the histamine H1 receptor, a series of experiments were conducted to address the role of this receptor in the control of energy homeostasis. The results of these experiments suggest four main findings. First, our detailed analysis of the metabolic phenotype of mice lacking H1r is consistent with previous findings in this strain of mice: the mice are obese, have elevated levels of leptin, and have a tendency for increased food intake during the early dark phase and for decreased energy expenditure. Expanding on what has been previously published (Morimoto et al., 1999, Mollet et al., 2001), we showed that all but very high doses of amylin and leptin do not suppress food intake in H1rKO mice. However, doses of amylin and leptin that do not alter feeding behavior were capable of inducing neuronal activation in key brain regions of H1rKO mice, similar to WT mice. Finally, our results indicate that while wild type mice show enhanced suppression of food intake when amylin and leptin were administered together, H1rKO mice do not.

4.1 Experiment 1: Metabolic phenotyping of H1rKO mice

The first aim was to better characterize the phenotype of the H1rKO mice. Previous studies (Morimoto et al., 1999, Mollet et al., 2001) had shown that H1rKO demonstrate a significantly higher growth rate and body weight compared to WT mice after 28 weeks of age, and exhibit higher plasma leptin and insulin levels by at least 48 weeks of age (Masaki et al., 2001b, Masaki et al., 2004). In our present study, however, it was observed that H1rKO mice had significantly higher body weight already by 18 weeks of age, and remained heavier for the following 6 weeks until the mice were sacrificed. This result is more consistent with the finding of our group by Mollet and colleagues that H1rKO mice already demonstrated significantly higher body weight by 12 weeks of age (Mollet et al., 2001).

In the present study, we also assessed if the levels of circulating hormones, which are known to reflect adiposity at least under weight stable conditions (Gloy et al., 2010), are altered accordingly in H1rKO mice at a time when H1rKO mice were more obese and when their body weight had reached 35-40g compared to 25-30g in WT mice. Amylin, leptin and insulin plasma levels were higher in H1rKO compared to WT mice, though the difference was significant only for leptin. These results are consistent with work from Masaki and colleagues who showed that H1rKO mice have elevated serum leptin levels and elevated ob mRNA expression in epididymal white adipose tissue (WAT) (Masaki et al., 2004). Though we did not measure glucose levels in our study, previous work suggests that mice deficient in H1r have a tendency for elevated serum glucose (Inoue et al., 1996, Masaki et al., 2004). Additionally, Masaki and colleagues showed that H1rKO also exhibit higher fasting serum levels of free fatty acids compared to WT mice at 48, but not at 12 weeks of age. Again, there is evidence in our study that the obesity in H1rKO might be aging related, but there is no clear consensus of when exactly obesity develops.

We also performed the first CT analysis of body composition in H1rKO mice; this was done at 19 weeks of age. H1rKO mice had significantly higher fat mass in both intraabdominal and subcutaneous compartments, which corresponds with elevated body weight and leptin levels in these mice. Again, these data support previous studies (Masaki et al., 2004), which had shown that H1rKO mice exhibit significantly higher body mass by the age of 48 weeks. In this report, higher body mass was accompanied by higher epididymal, mesenteric, and retroperitoneal WAT mass, and increased triglyceride concentrations in the liver and skeletal muscle. Furthermore, the adipocytes in epididymal WAT were histologically larger and the amount of fat deposition in brown adipose tissue (BAT) was greater in H1rKO mice than in control mice.

A factor that plays an important role in energy homeostasis is BAT and its key protein uncoupling protein 1 (UCP-1) (Nicholls and Locke, 1984, Boss et al., 1997, Fleury et al., 1997). The gene expression of the UCP family is regulated by humoral and neuronal factors (Gong et al., 1997, Masaki et al., 1997, Zhou et al., 1997, Hidaka et al., 1999, Masaki et al., 1999); e.g. leptin, administered centrally, upregulates the gene

expression of the UCP family (Cusin et al., 1998). Interestingly, Masaki and colleagues described that the expression of UCP-1 mRNA was lower in 48-week-old H1rKO mice compared to WT mice of the same age (Masaki et al., 2004). This might be related to the leptin resistance and hence lack of leptin induced upregulation of UCP-1 in these mice. Further, it seems plausible that the signal transduction between leptin and histamine neurons is involved in the central regulation of the UCP expression (Masaki et al., 2001b). H1 receptors are directly involved in the central control of energy homeostasis through sympathetic influences on UCP expression in BAT (Masaki et al., 2001a, b). For example, when methyl (2-[2-pyridyl]ethyl)amine dihydrochloride, a H1-receptor agonist, was administered for seven days into the lateral cerebroventricle or intraperitoneally, UCP expression in BAT was increased in WT but not in H1rKO mice. Thus the lack of H1 receptors, with a concomitant decrease of UCP expression, might contribute to the development of obesity in older H1rKO mice. The potential direct and causal link to leptin resistance in these mice remains to be tested.

Previous studies had shown that H1rKO mice exhibit increased locomotor activity, especially during the early portion of the light phase, with decreased activity during the dark phase (Inoue et al., 1996). However, this study reported that total activity over 24 hours was not significantly different. Consistent with these results, we also did not detect a statistical difference between wild type and H1rKO mice when physical activity was measured by counting the number of beam breaks in the animals' home cage over a 24-hour period. We did not measure hourly activity; therefore, we can only speculate that a difference between the genotypes in the early portion of the light phase would emerge if we were to analyze the data hour-by-hour.

Moreover, the H1rKO mice showed significantly decreased ambulation and time spent rearing for the first 30 minutes in a new environment (Inoue et al., 1996). Hypothalamic histamine is released in rats forced to swim for one hour (Endou et al., 2001), suggesting that histamine may have been released because of the stress or, alternatively, because the rats are performing a motivated behavior to survive. This could be an explanation why H1rKO show less activity in a new environment and

also are less prone to stress during handling, as it was seen when our mice were handled in the present study.

We also assessed, for the first time by means of indirect calorimetry, if there were differences in energy expenditure between wild type and H1rKO mice during the light and dark phase. While not significant, we observed a tendency toward lower total energy expenditure in H1rKO mice. Because we saw no difference in physical activity, the primary cause of positive energy balance in H1rKO mice still remains to be determined. Measurement of core body temperature in combination with BAT activity (see above) and diet-induced thermogenesis will shed additional light on this matter. Leptin administration is a known activator of energy expenditure and selectively promotes fat metabolism in ob/ob mice (Hwa et al., 1997); thus decreased energy expenditure in H1rKO mice might directly result from decreased sensitivity to endogenous leptin in these mice.

Finally, indirect calorimetry was also used to measure the respiratory quotient of wild type and H1rKO mice during both the light and dark phase in order to determine the primary source of energy utilized over a 24-hour period. We observed no difference between genotypes, suggesting that on average, both genotypes pull from similar energy sources (carbohydrate, fat, or protein) over the course of a day.

In our study we observed that *ad libitum*-fed H1rKO mice have a tendency toward increased food intake in the first hours after dark onset, and that they also consumed more food during the first few hours of the dark phase following a 6-hour fast ($p<0.001$). It is possible that this is the result of decreased sensitivity to satiation hormones like amylin during the early dark phase; i.e. when rodents typically ingest their biggest meal and when the amylin effect therefore may be most pronounced. This notion is supported by the fact that the H1rKO mice were indeed more resistant to certain doses of exogenous amylin and leptin, compared to wild type controls (Mollet et al., 2001). Despite these observations during the early dark phase, neither total 24-hour cumulative food intake, nor total light or dark phase intake, were different across genotypes, regardless of whether mice had been fasted. This suggests

that differences observed during the initial dark phase are compensated for; the underlying mechanisms are unknown.

Our data are consistent with previous work (Masaki et al., 2001b) showing no change in total 24-hour food intake; however, this pattern does appear to shift with age, as H1rKO mice at 48 weeks of age did consume more food than wild type mice, primarily during the light phase (Masaki et al., 2004). Because 24-hour food intake did not differ significantly between H1rKO and WT mice at this age in the present study, the higher body weight in H1rKO was likely due to lower energy expenditure, though the exact mechanism by which energy expenditure is altered remains to be established. Due to the known effects of amylin (Mack et al., 2007, Osaka et al., 2008, Wielinga et al., 2010) and leptin on energy expenditure (Halaas et al., 1995, Halaas and Friedman, 1997, Hwa et al., 1997, Widdowson et al., 1997), it seems plausible that the reduced responsiveness of H1rKO to amylin and leptin may be involved.

Previous studies have shown that a disturbed circadian feeding rhythm per se may result in obesity (Beck et al., 1990, Fukagawa et al., 1992, Murakami et al., 1995, Arble et al., 2010). For instance, studies done in obese (fa/fa) Zucker rats showed that the hyperphagia of these rats is characterized not only by the increased quantity of ingested food, but also by a redistribution of the meals during the light-dark cycle (Beck et al., 1992). This may be partially explained by the fact that Zucker rats have increased NPY concentrations within the suprachiasmatic nucleus of the hypothalamus, which is considered the major controller of the circadian feeding rhythm (Beck et al., 1992). Fukagawa and colleagues also showed that with the progression of age and obesity, obese Zucker rats gradually exhibit disrupted patterns of nocturnal feeding, drinking, and ambulation. More specifically, these obese rats eat larger meals during both the dark and light phase than their controls. Additionally, obese rats eat more frequently during the light phase, and also show increased ambulation during the light phase with progression of age (Fukagawa et al., 1992). Whether similar phenomena are present in H1rKO mice remains to be studied in detail.

To summarize, while the exact mechanism causing obesity in H1rKO mice still needs further elucidation, it is clear that H1rKO mice are less sensitive to the eating inhibitory effects of amylin and leptin. It was previously established that amylin (5 and 20μg/kg) and leptin (1.3mg/kg) administration in H1rKO mice induces a markedly attenuated anorectic effect (Morimoto et al., 1999, Mollet et al., 2001). However, it had not been previously tested how these animals behave after the administration of higher doses of amylin or leptin, and whether central neuronal activation patterns are altered following their administration in H1rKO mice. We therefore investigated the feeding behavior and central neuronal response pattern in H1rKO mice after the administration of different doses of amylin and leptin to determine if the decreased amylin and leptin sensitivity in H1rKO mice is a result of faulty receptor functioning or a disruption in the networks downstream of the receptors.

4.2 Experiment 2: Do mice lacking H1 receptors respond to anorectic doses of amylin?

By assessing different doses of amylin (5, 20, 50, 200μg/kg), we observed that H1rKO mice are less responsive than wild type mice to all doses of amylin, except the highest dose (200μg/kg) tested, which caused a similar reduction in food intake in both genotypes. While the lack of functional H1 receptors appears to be a clear contributor to decreased amylin sensitivity, other factors, like dysfunctional amylin receptors or even obesity itself, might also lead to decreased amylin sensitivity. Indeed, diet-induced obese (DIO) rats show a somewhat attenuated response to amylin after long term exposure to a high energy diet (Boyle et al., 2011).

To determine indirectly if a disruption in amylin receptor function underlies decreased amylin sensitivity in H1rKO mice, amylin-induced cFos activation was assessed in the AP of wild type and H1rKO mice. The immediate early gene product cFos acts as a marker of neuronal activation. Despite the differential effect on food intake, both WT and H1rKO mice showed significantly increased cFos activation in the AP following amylin treatment (50μg/kg); interestingly, H1rKO mice showed an even higher activity than WT mice. Our results demonstrate that the responsiveness of

amylin's primary target brain area seems to be intact in H1rKO mice, and that the sensitivity of the AP to amylin is potentially even higher in H1rKO mice than in WT mice. H1rKO mice only reacted to a very high dose of amylin (200µg/kg) with a reduced eating response, which could have non-specific effects on receptors other than the CT-R. These results lead us to suggest that the lack of amylin's effect on food intake in H1rKO mice most likely lies downstream of the amylin receptor in the AP, and that the effect observed when high doses of amylin were given potentially lead to the activation of additional or unspecific pathways to reduce food intake. The overall low number of cFos-positive cells suggests that additional optimization of the cFos staining protocol for mice is required. Therefore, we view this cFos study as a preliminary experiment that needs to be repeated.

As previously discussed, the AP is functionally linked to several other brain regions, including the LPBE, NTS, CEA, and the BSTL (Rowland et al., 1997, Riediger et al., 2004). Dense anatomical projections also exist from amylin activated neurons in the LPBE to the VMH and the ARC (Potes et al., 2010). The existence of such neuronal connections might explain why the AP can be still activated by amylin in H1rKO mice and that the reduced amylin sensitivity may exist in downstream projections. It seems possible, for example, that VMH-mediated signaling is diminished in H1rKO mice, since we know that the H1 receptors are mainly expressed within the VMH (Palacios et al., 1981). Thus the link between AP, LPB and VMH is probably a very important starting point for future studies. Furthermore, it has been proposed that amylin may be able to trigger histamine release which can act on hypothalamic histamine H1-receptors (Mollet et al., 2003). This is supported by findings that the monkey hypothalamus contains a discrete number of amylin-immunoreactive neurons which may be involved in amylin's effect on food intake via the histaminergic system (D'Este et al., 2001).

4.3 Experiment 3: Do mice lacking H1 receptors respond to anorectic doses of leptin?

By assessing different doses of leptin (500µg/kg, 1, 5 and 10mg/kg), we showed here that H1rKO mice are less responsive to leptin treatment unless high doses (5 and 10mg/kg) were administered. Yet, similar to what we observed with amylin-induced

cFos expression in the AP, leptin treatment (1.3mg/kg) induced a significant increase in pSTAT3 activation within the ARC and VMH of the hypothalamus in both WT and H1rKO mice. Again this suggests that the responsiveness of primary leptin receptors in the hypothalamus is still present in H1rKO mice.

It is well established that chronic high-fat feeding and hyperleptinemia are associated with decreased pSTAT3 signaling within the hypothalamus in rats (El-Haschimi et al., 2000, Roth et al., 2008). However, this does not seem to be the case for H1rKO mice despite the lack of a functional leptin response; although H1rKO mice have higher basal plasma leptin levels than WT mice, both genotypes mounted a similar pSTAT3 response following leptin treatment. Due to the attenuated eating effect after low to moderate leptin doses, it is suggested that H1rKO mice are leptin resistant when more physiological concentrations of leptin are circulating within the blood.

As another possible explanation of the leptin resistance in H1rKO, there is evidence that elevated blood leptin levels in itself may overstimulate leptin receptors, which leads to an upregulation of negative feedback pathways and which blocks further leptin signaling (Knight et al., 2010). Leptin stimulates the expression of SOCS-3, a protein that directly inhibits leptin signaling in the ARC (Enriori et al., 2007) and that acts as a negative regulator of pSTAT3 signaling in the hypothalamus. Leptin resistance is therefore associated with elevated SOCS-3 levels in the ARC (Munzberg et al., 2004). It might be possible that SOCS-3 is involved in the decrease of leptin's effect on food intake in H1rKO mice in some form, but these mice are still responsive to higher leptin doses. High leptin doses might overcome the leptin resistance perhaps due to an activation of other pathways that lead to a decrease of food intake. Hence, studies need to be done to investigate the SOCS-3 levels in the ARC in H1rKO mice after different doses of leptin.

Overall, we suggest that the dysfunction of leptin's effect most likely reflects the lack of histamine H1 receptors, specifically in the VMH. This is supported by the fact that hypothalamic histamine release is increased after leptin administration (Yoshimatsu et al., 1999, Morimoto et al., 2000). It is likely that leptin also induces histamine release in H1rKO mice, but it cannot exert its effects on food intake and energy expenditure

because H1 receptors are absent.

4.4 Experiment 4: Do mice lacking H1 receptors respond to combined amylin – leptin administration?

As we and others have shown that H1 receptors are involved in amylin and leptin action (Morimoto et al., 1999, Morimoto et al., 2000, Mollet et al., 2001), the aim of the final part of our experiments was to investigate the effect of combined administration of amylin and leptin in H1rKO mice; namely whether this combination may also be effective in mice lacking H1 receptors.

In fact, the effect of a combined treatment was only seen in WT mice. Amylin plus leptin treated WT mice, but not H1rKO mice, ate significantly less compared to the control saline-saline group. Interestingly and unexpectedly, the amount of food eaten was not significantly lower in WT mice treated with either amylin or leptin alone, as was observed in Experiments 2 and 3 where similar doses were administered. We speculate that this may have been due to the long stay of the mice in the metabolic cages. The animals were kept for more than two months under the conditions of individual housing in a non-diversified environment with repeated peripheral injections. In fact this resulted in a decreased amount of food intake in all groups when compared to the basic measurements of Experiment 1. However, other studies reported that amylin reduces food intake in rats (Lutz et al., 1994) and mice (Morley and Flood, 1991) after repeated peripheral or central administration and even during chronic administration in rats (Arnelo et al., 1996, Lutz et al., 2001).

The main result regarding eating behavior in H1rKO mice was that the combined amylin-leptin administration did not induce a significant reduction in food intake compared with the saline-saline treated animals. These findings support our suggestion that amylin's effect of restoring leptin responsiveness may depend on an intact histaminergic system; this would therefore explain why this reinforcing effect did not occur in mice lacking H1 receptors.

Although we did not test a cFos response in this experiment, we suggest that H1rKO mice probably have elevated cFos reactivity after amylin–leptin treatment, as it was the case after amylin alone in Experiment 2. We rather presume that the critical site of interaction is the hypothalamus. As mentioned, previous studies suggested that amylin interacts with leptin at the level of the VMH and the ARC; amylin pre-treatment increases leptin sensitivity in lean rats (Turek et al., 2010) and partially restores hypothalamic leptin sensitivity in leptin resistant DIO rats by reinstating normal leptin induced pSTAT3 activity in the VMH (Roth et al., 2008). However, future studies are required to test this hypothesis specifically (see section 4.5).

We conclude that the histamine H1 receptor is involved in the feeding inhibitory effects of amylin and leptin treatment. Future studies will be necessary to prove the hypothesis that H1 receptors are required specifically for the amylin-leptin interaction.

4.5 Outlook and future studies

Further studies are needed to test in more detail our hypothesis that histamine plays a specific role in the amylin-leptin interaction. The results presented in this work only show that amylin and leptin are individually less active in H1rKO than WT mice. The lack of an eating inhibitory effect of the amylin-leptin combination treatment in H1rKO mice could simply be due to an abolishment of these individual effects, rather than of their interaction.

The role of H1 receptors in this interaction will be tested based on the studies by Roth et al. (2008) and Turek et al. (2010). For example, future experiments will test whether amylin potentiates the leptin induced pSTAT3 response in the VMH in mice, whether amylin increases leptin receptor binding in the ARC and the VMH, and whether amylin increases leptin receptor mRNA expression in the hypothalamus. If our hypothesis were correct, all these effects would only be observed in WT, but not in H1rKO mice. Similar experiments will test whether leptin enhances amylin-induced cFos expression in the AP (Roth et al., 2008) in WT but not in H1rKO mice.

However, it is important to note that a primary role of the AP in the amylin-leptin interaction seems less likely than the involvement of the VMH (Turek et al., 2010).

To investigate the involvement of histamine in the VMH, it will also be worthwhile to investigate whether amylin, leptin or their combination trigger histamine release in the hypothalamus, and whether this response differs between WT and H1rKO mice. Finally, other brain areas, such as the PVN, which lie outside the primary ARC or VMH target sites, need to be investigated by assessing amylin- and leptin-induced neuronal markers, since there is evidence that complex neuronal connections do exist between these different brain areas (Palacios et al., 1981, Elmquist et al., 1998, Elmquist et al., 1999).

Another experimental approach to test our hypothesis might be to design similar experiments using rats and to induce a blockade of H1 receptor in the VMH by central administration of H1 receptor-antagonists or virus-induced H1 receptor knockdown, in combination with amylin and leptin treatments. This approach would give us the possibility to analyze further parameters of energy expenditure (e.g. diet-induced thermogenesis, body temperature in multiple locations) and to very specifically target the H1 receptor in the VMH, without the potentially confounding developmental effects in the complete H1rKO mice.

Finally, to overcome some technical limitations of the present studies, some experiments will require repeating, such as the combined amylin-leptin treatment experiments, to increase group size and statistical power. Also, since there were some technical issues with the cFos staining procedure in our mice, we decided not to count the cFos activated cells in the Experiment 4. This problem must be resolved for future studies. Various perfusion and immunohistochemical protocols from other laboratories should be tested. Preliminary tests examining the influence of various perfusion protocols on brain tissue show promising results.

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6 Acknowledgements

I am grateful for the financial support of the Swiss National Science Foundation and the Novartis Foundation, which enabled this project.

My particular thank goes to:

Dr. Christina Neuner Boyle,
who supported me in all situations with much patience and effort, by being a wonderful person and doing a great job as a supervisor.

Prof. Dr. Thomas Lutz,
who gave me the opportunity of writing this thesis in his group and who always was there for any advice and his critical examination of this work.

PD Dr. Colin Schwarzwald,
for his critical reading of my thesis.

PD Dr. Thomas Riediger,
who helped much with his IT knowledge and his advices for study designs.

Dr. Viktoria Gloy,
who was assisting me with the CT scan procedure.

Sara Benz, Lettebrhan Ghebre and Josiane Grob,
who all did a great job by taking care of the animals.

Thanks a lot to my office colleagues for the help and for making life easier and fun:
Catarina, Dani, Daniela, Lena, Lori, Melania, Mélanie, Miriam, Nadine, Kathrin, Kerstin, Sarah and Tito.
A special thank to **Stefan**, who helped as much as possible and who encouraged me during these years, and thanks to my family for any support and being there for me.

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